Influence of nutrition on parasitism in periparturient dairy ewes

Thesis

Influence of Nutrition on Parasitism in Periparturient Dairy Ewes

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by

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DECLARATION

This is to declare that this thesis has been composed by myself, and has not been accepted in any previous application for a degree at any other institution. The work has been completed by me and all sources of information have been acknowledged by means of references.

Elizabeth C. Partington

ABSTRACT

Influence of Nutrition on Parasitism in Periparturient Dairy Ewes

Increased anthelmintic resistance has lead many researchers to investigate alternative methods of parasite control. It is believed that most larval contamination of the pasture is derived mainly from the mature breeding ewe due to the periparturient rise (PPR) in faecal egg counts (FECs). The aim of this study was to determine if manipulating the dietary supply of metabolisable protein (MP) or fish oil to the periparturient ewe can moderate the PPR.

The first experiment investigated the effects of increased MP supply and fish oil on the PPR of machine-milked ewes. The second experiment investigated the effects of nematode infection and increased MP supply on the PPR of machine-milked ewes and the third experiment investigated the effects of machine milking compared to suckling twin lambs and the effect of increasing dietary MP on the PPR.

In all the experiments, FECs, peripheral eosinophil counts, blood haematology and metabolite analyses were carried out and milk yields and composition were recorded. Additionally, in Exp.1 immunoglobulin titres were determined, in Exp.2 pepsinogen assays were carried out and in Exp.3 blood was collected for lymphocyte stimulation responses. In Exps.2 and 3, the ewes were slaughtered to investigate nematode burden, mucosal mast cell (MMC) and mucosal eosinophil counts.

The dietary treatments had no significant effects on the immunological parameters throughout all three experiments, although in Exp.2 larval challenge increased MMCs, mucosal eosinophils and pepsinogen assays. There was no benefit of increased dietary MP or fish oil on FECs in Exp.1. and no beneficial effect of increased dietary MP on FECs during Exp.2. However, in Exp.3 increased MP reduced the FECs from the twin suckled ewes but had no effect on the machine-milked ewe FECs. The machine-milked ewes had significantly lower milk yields than the suckled ewes and it may be that the machine-milked dairy ewe may not suffer the PPR due to some unidentified mechanism.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
βHB	Beta Hydroxybutyrate
BMP	Basal Metabolisable Protein
BMS	British Milk Sheep
BSE	Bovine Spongiform Encephalopathy
$CD4^+$	Helper T lymphocyte subset
CI	Confidence Interval
CLA	Conjugated linoleic acid
Con A	Concanavalin A
CP	Crude Protein
CS	Condition Score
DHA	Docosahexaenoic acid
DM	Dry Matter
DMSO	dimethyl sulphoxide
DP	Digestible protein
DUP	Digestible Undegradable Protein
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaeionic acid
EPG	Eggs Per Gram of faeces
ERDP	Effective Rumen Degradable Protein
FA	Fatty Acid
FECs	Faecal Egg Counts
FME	Fermentable Metabolisable Energy
GC	Gas chromatographer

GIN	Gastrointestinal nematodes
HMP	High Metabolisable Protein
Ig	Immunoglobulin
IL	Interleukin
IPM	Integrated pest management
L_3	Infective stage larvae
LMP	Low Metabolisable Protein
LW	Live weight
ME	Metabolisable Energy
MMC	Mucosal Mast Cell
MP	Metabolisable Protein
MTT	3-(4-5-dimethyldiazole-2-yl)-2,5-diphenyl tetrazolium bromide
NDF	Neutral detergent fibre
OD	Optical Density
PBS	phosphate buffered saline
PCV	Packed Cell Volume
PE	Petrol Ether
PFA	Paraformaldehyde
PGE	Parasitic gastro-enteritis
PPR	Peri-parturient Rise in faecal egg counts
PUFA	Polyunsaturated Fatty Acids
PWM	Pokeweed Mitogen
RSM	Rape Seed Meal
SED	Standard error of the differences between the mean
SBM	Soya Bean Meal
SNF	Solids non Fat
TCM	tissue culture medium

Th1	$CD4^+$ helper T lymphocyte polarised subset 1
Th2	$CD4^+$ helper T lymphocyte polarised subset 2
WBC	White Blood Cell

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INTRODUCTION

Ewes previously immune to parasite infection show an increase in faecal nematode egg output around parturition, as a result of a periparturient breakdown of resistance to gastrointestinal nematodes – termed the periparturient rise (PPR) (Connan, 1976). Parasitic larvae derived from the ewe at this time, together with those that have survived over winter, are the major source of gastrointestinal nematode infection for young lambs (Jackson *et al.*, 1988). Additionally, drug resistance in gastrointestinal nematodes is increasing and has been recorded against all three classes of anthelmintic compounds (Barrett *et al.*, 1998) and the prevalence of anthelmintic resistance in the world is still increasing (Borgsteede *et al.*, 1996). These developments and growing concerns over current farming methods, including the possibility of chemical residues in animal products (Waller, 1993), and the emergence of bovine spongiform encephalopathy (BSE), have called for the investigation of more sustainable approaches to controlling nematode infections.

Previous studies have shown that the provision of fishmeal in the periparturient period reduced faecal egg output (Donaldson *et al.*, 1998; 2001). Within the overall hypothesis that the PPR has a nutritional basis, this study aims to determine whether an increased intake of metabolisable protein (MP), other than that found in animal/fish products, i.e. fishmeal, and/or fish oil, can affect the expression of ewe immunity to *Teladorsagia circumcincta*. The nematode *T. circumcincta* is a major cause of parasitic gastro-enteritis (PGE) in sheep in the temperate areas of the world (Urquhart *et al.*, 1996).

Most work on the PPR has involved ewes which suckle their young. Donaldson *et al.* (1998; 2001) studied both the effects of protein and energy supply and the effects of lamb number suckling on the ewe and found that an increased supply of fishmeal protein reduced nematode egg output and those ewes suckling twins and triplets had higher nematode egg output than those suckling single lambs. This study therefore aimed to

determine the effect of whether a non-animal source of metabolisable protein on nematode egg output using dairy ewes that, according to Cant *et al.* (2001), would be under a great production pressure. Machine milking would facilitate the quantification of milk production more than when suckling lambs, offering an insight into the effects of nematodes on ewe production and *vice versa*.

1.1. Teladorsagia circumcincta *life cycle and importance*

Gastrointestinal parasitism remains a major factor limiting the production of ruminants and has adverse effects on their welfare (Zajac *et al.*, 2000; Athanasiadou *et al.*, 2000). One of the most relevant ovine parasites known to be a major cause of Parasitic gastro-enteritis (PGE) in the temperate areas of the world is *Teladorsagia circumcincta* (*T. circumcincta*), formerly known as *Ostertagia circumcincta* (Urquhart *et al.*, 1996). This slender reddish-brown worm, between 0.7 and 1.2 centimetres long is found on the surface of the ovine abomasal mucosa (Kassi, 1999; Urquhart *et al.*, 1996). It has a simple life cycle, beginning with adult male and female worms in the abomasum of the host. Fertilised eggs are passed in the faeces, they are oval, thin-shelled, colourless, medium-sized (60-110μm) eggs containing 8-32 blastomers (Kassai, 1999).

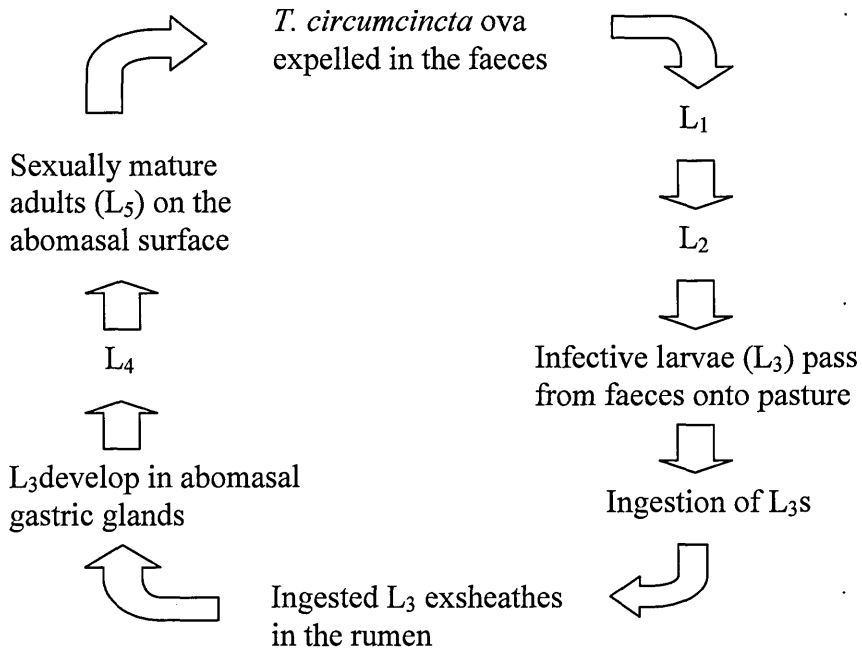


Figure 1.1. – *The direct life cycle* Teladorsagia circumcincta *(adapted from information in Urquhart* et al., *1996; Kassai, 1999)*

The eggs hatch and larval development begins within the protective environment of the faeces. Under optimal conditions of moisture and temperature (22-26°C) the larvae can reach the infective (L_3) stage in approximately 5-6 days (Stromberg, 1997). The larvae migrate into the neighbouring forage with the help of forces such as rain and await ingestion (Stromberg, 1997). Upon ingestion the larvae undergo an additional two moults to the adult stage which takes approximately 18 days excluding hypobiosis occurring (arrested development at the early 4th larval stage for periods of up to 6 months) (Urquhart *et al.*, 1996) (Figure1.1). Kassai (1999) reports that the cattle parasite *Ostertagia ostertagi* adults die within two months. Armour *et al.* (1966) found that the majority of a dose of 100,000 *T. circumcincta* larvae given to 6-month-old sheep were in the gastric glands by 4 days of infection and adults by 8 days, a proportion remained arrested but most had resumed development by day 60 and the adult population began to decrease from day 16 onwards.

This parasite is a remarkably successful parasite and major constraint on sheep production in temperate areas of the world (Urquhart *et al.*, 1996). It is more successful at colonising sheep than other parasites such as *Trichostrongylus axei*, *Trichostrongylus vitrinus* and *Cooperia spp.* (Stear *et al.*, 1999). This may be due to its greater fecundity or its ability to survive the winter, as either inhibited larvae (hypobiosis), or by surviving on the pasture (Stear et al., 1999). In an experiment by Stear *et al.*, (1997), over 80% of the natural burden of worms in over 500 lambs in Scotland were *T. circumcincta.*

Clinical signs of infection with *T. circumcincta* can be inappetance, weight loss and diarrhoea (Urquhart *et al.*, 1996). Larval challenge can cause severe pathophysiological disturbances even in an immune ewe, for example elevated pepsinogen levels, altered albumin catabolism and increased losses of plasma protein (Yacoob *et al.*, 1983). The severity of symptoms can be dependant on factors such as size of burden (a heavy infection would comprise of 40,000 or more adults in the abomasum (Urquhart *et al.*, 1996) and host age, as well as immunological and nutritional status of the host (Brunsdon, 1982).

Teladorsagia circumcincta compromises the abomasal function by causing a change in endocrine and enzyme secretion and increasing the pH from 2.0 to 7.0 (Sykes, 1994; Urquhart *et al.*, 1996; Bowman, 1999). The increased neutrality of the gastric juice results from the larvae developing in the gastric glands, there is a decrease in functional gastric gland mass (Urquhart *et al.*, 1996). The gastric glands are responsible for the production of the highly acidic proteolytic gastric juice, especially the parietal cells which are responsible for the production of hydrochloric acid. The increased pH leads to a failure to activate pepsinogen to pepsin and this leads to a failure to denature proteins, an increased permeability of the epithelium to macromolecules and an increased level of pepsinogens in the plasma (Urquhart *et al.*, 1996).

Although some protein is reabsorbed, leakage of endogenous protein leads to a disturbance in postabsorptive nitrogen and energy metabolism (Knox and Steel, 1996). This may be due to an increased demand for the synthesis of proteins such as albumin, immunoglobulins and structural liver proteins, which occur at the expense of skeletal muscle, wool and milk protein synthesis (Knox and Steel, 1996; Urquhart *et al.*, 1996). Even a sub-clinical burden with *T. circumcincta* can lead to production losses, such as poor feed conversion and suboptimal weight gain in lambs (Coop *et al.*, 1977), reduced production in adult sheep (Yakoob, 1983) or reduced milk production and wool growth/quality in lactating ewes (Leyva *et al.*, 1982).

1.2. Current methods of controlling nematode infections in sheep

The risk of exposure to parasites to the efficiency of production is considered very high and is recognised in the intensity of anthelmintic use in production systems around the world (Sykes, 1994). Currently the principle method employed in the control of nematodes in domestic animals is chemoprophylaxis (Roos, 1997). Many industries rely on effective chemically-based parasite control for reducing the economic cost of parasitism and the animal suffering caused by parasites (Sangster, 2001). Even in a survey of 90 UK 'organic'

sheep systems, the majority (64%) of the farmers used chemical anthelmintics to varying degrees (Roderick *et al.*, 1999) with only 6% using homeopathic remedies and only 2% responding that they had no control policy for nematodes. Many used a combination of anthelmintics and other practices (Roderick *et al.*, 1999).

Hennessy (2000) suggested that chemoprophylaxis is, and probably will continue to be, the cornerstone in livestock parasite treatment and our dependence on it has long since passed the point of no return. Hennessy (2000) also suggested that chemical intervention in parasitic disease is here to stay and to suggest that it can be eliminated is fallacious. However, the combination of anthelmintics, along with management practices, may be combined to reduce the 'over-use' of chemical treatment. For example, Barger (1999) reported that dosing young animals and then moving, "dose and move," them onto "clean" pasture (pasture which has not been grazed by that species in that grazing season) is, at present, a common practice in small ruminant production. This practice is usually provided for nutritional as well as parasitological reasons. However this practice may even encourage anthelmintic resistance to develop (Coles, 2002).

1.3. Concerns about current control methods

1.3.1. *Past and present anthelmintic drugs*

Several methods of controlling parasites have been investigated but the most frequently used is chemotherapy (Roos, 1997). The historical use of drugs to control anthelmintic infection has been reviewed by Gibson (1975). The drugs used in the first half of the 20th century were often plagued with problems such as poor efficacy, being effective against only one worm species or had toxic effects on the host animal. They involved hazardous concoctions of copper, arsenic and nicotine with unknown modes of action (Bennet-Jenkins and Bryant, 1996). These compounds were the only anthelmintics available until the marketing of the first, true, broad-spectrum anthelmintic phenothiazine, in the late

1930s, and, later in the 1960s, thiabendazole with much better broad-spectrum activity and safety levels (Waller, 1999).

Currently there are three chemical classes of anthelmintic drugs on the market; benzimidazoles, imidothiazoles and the macrolytic lactones (Condor and Campbell, 1995). Within a class, the compounds share features of chemical structure and mechanisms/sites of action (Sangster, 2001). Drugs listed for use in sheep include, fenbendazole, thiabendazole (Gibson, 1975) and albendazole, (benzimidazoles), levamisole and morantel (Gibson, 1975) (imidothiazoles) and ivermectin, moxidectin, avermectins and milbemycins (macrocyclic lactones), introduced in 1988 (Williams, 1997).

The benzimidazoles bind to the nematode β-tubulins which alter the tubulin-microtubule equilibrium causing the depolymerisation of microtubules (Lacey, 1988; McKellar, 1997; Sangster *et al.*, 2002; Sangster and Dobson, 2002). They are also known to inhibit fumarate reductase and glucose transport in nematodes (McKellar, 1997). Levamisole and morantel bind to nematode acetycholine (Ach) receptors by mimicing this neurotransmitter (a cholinergic agonist), this leads to a permanent opening of the ion channels, causing spastic contraction of the worms, more specifically levamisole is thought to also act at nematode nicotinic neuromuscular receptors (Sangster, 1999). Macrocyclic lactones, also known as avermectins and milbemycins are produced as fermentation products by the actinomycete *Streptomyces avermitilis* in culture (Kettle, 1982). They appear to act on glutamate gated chloride channels, binding to the α-subunit, irreversibly opening the chloride channels in the muscle membranes of the pharynx, and possibly the somatic musculature, causing either fatal starvation and/or paralysis (Conder and Campbell 1995; McKellar, 1997; Sangster, 1999; Sangster and Dobson, 2001; Sangster *et al.*, 2002).

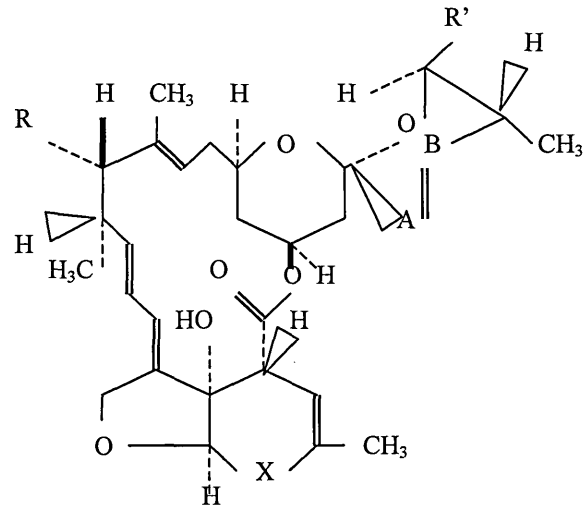


Figure 1.2. *The structure of the macrocyclic lactone (the letters R, R', A, B and X lead to the different molecule structures which comprise the different members of the macrocyclic lactone class.*

1.3.2. *The possibility of commercially available, new anthelmintic drugs*

Geary *et al.* (1999) reported that there were only 3 new classes of drug that hold any promise for veterinary applications; diketopiperazines (paraherquamides and marcfortines), cyclic depsipeptides (PF1022A) and nitazoxanide. Geary *et al.* (1999) also reported that there was no information that any compound from these classes was in clinical development. Indeed, it seems unlikely that PF1022A and nitrazoxanide will make it into the ruminant health market in the near future. Diketopiperazine/paraherquamides have fewer limitations associated with them, apart from the possible unknown effect of residues left in the host tissues/produce. Studies on the effects of paraherquamide on sheep (Shoop *et al.*, 1990) and on cattle (Shoop *et al.*, 1992) parasites have had favourable results, most of which are summarised in Geary *et al.* (1999). However, they will find it hard to compete in the commercial market, with the macrocyclic lactones as they have no effects against arthropods and cestodes.

Natural plant-derived substances have also been investigated. For example, Bennet-Jenkins and Bryant (1996), studied the anthelmintic effects that endogenous growth regulators (G3) from *Eucalyptus spp.*, which are endoperoxides (containing free radicals), and claimed that

they offer a novel source of compounds worthy of testing for anthelmintic properties. Bennet-Jenkins and Bryant (1996) found that feeding fresh *Eucalyptis grandis* leaves to goats had an anthelmintic effect against *Haemonchus contortus* but not *T. circumcincta.*

There are few signs of new anthelmintic products being developed, very few animal health companies are committed to the discovery of new antiparasitic drugs, and any compound which is currently receiving serious research and development consideration will not be commercially available for several years (Geary and Thompson, 2003).

1.3.3. *The increasing concerns and current status of anthelmintic resistance*

Conder and Campbell (1995) described resistance as 'a heritable reduction in the sensitivity of a parasite population to the action of a drug, the reduction being expressed as a decrease in the frequency of individual parasites affected by exposure to the drug (in comparison to the frequency observed in the same population upon initial or previous exposure)'. In other words, a shift occurs in the susceptibility to a drug, usually first recognised as a failure to control parasitism (Sangster, 2001; Sangster and Dobson, 2002). There are a number of different types of resistance such as side resistance (when the nematodes are resistant to all drugs in a chemical class), and multiple resistance (when the nematodes are resistant to 2 or more chemical classes of drug). Reversion is used to relate to the loss of resistance back to susceptibility, which is a slow if not unlikely occurrence (Leathwick *et al.*, 2001).

Resistance develops slowly at first then escalates to maximum levels (when treatment failure occurs) relatively quickly (Sangster, 1999). It is likely that there are resistant genes present in populations of nematodes and the nematodes which survive treatment reproduce and pass on alleles that confer resistance to their offspring (Sangster, 1999). There are thought to be three stages for the development of resistance in a nematode population: firstly, there is a low frequency of resistant individuals; then, following selection, there is a

stage where heterozygous resistant individuals predominate and then, finally, a resistant phase where homozygous resistant individuals predominate (Pritchard, 1990).

Unfortunately resistance is virtually ubiquitous (Sangster *et al.*, 2002); anthelmintic resistant nematodes have been recorded in sheep, goats and horses and more recently in cattle (Coles, 2002). The ovine nematodes of major importance, *T. circumcincta*, *H. contortus* and *Trichostrongylus* spp., have been found resistant to the three broad spectrum groups, mainly the benzimidazoles and the levamisole/morantel groups throughout the sheep and goat producing regions of the world, and the macrocyclic lactones are now under severe threat, particularly in South America (Waller and Larsen, 1996). Indeed, a recent survey of sheep farms in southern Australia found that only 9% of farms had parasites that were not resistant to any drug and in Paraguay about 75% of farms had nematodes resistant to all 3 chemical classes (Gill and LeJambre, 1996). Jackson *et al.* (1992) reported the first evidence of ivermectin resistance in goats and the first case of multiple anthelmintic resistance in the UK. Not only has resistance occurred to all major drug classes, in several species of parasite, but several isolates are resistant to all 3 major drug classes (Sangster, 2001). Evidence of this has also been recorded in the UK in sheep (Sargison *et al.*, 2001; Yue *et al.*, 2003; Bartley *et al.*, 2004).

Table 1.1. Reported resistances to anthelmintic classes in parasitic helminths

Anthelmintic class	Host *spp.*	Country	Reference
Benzimidazoles	many	many	(Conder and Campbell, 1995)
Imidothiazoles	Sheep	Cuba	(Arece *et al.*, 2004)
	many	many	(Conder and Campbell, 1995)
Macrocyclic lactones	Sheep		(Shoop, 1993)
	many	many	(Conder and Campbell, 1995)
	Sheep	South Africa	(Van Wyk and Malan, 1988)
Macrocyclic lactones/	Sheep	Paraguay	(Gill & Le Jambre, 1996)
Benzimidazoles & imidothiazoles	Sheep	UK	(Sargison *et al.*, 2001)
(Multiple resistance)	Sheep	UK	(Yue *et al.*, 2003)
	Goats	UK	(Jackson *et al.*, 1992)
	many	many	(Condor and Campbell, 1995)

Conder and Campbell (1995) provided a comprehensive list of resistance whilst Sangster and Dobson (2002) summarised more recent findings.

Regions such as Australia and South America that are noted for their intensive livestock industries, have the highest levels of resistance (Sangster *et al.*, 2002). Evidence in the UK and Europe suggests that the development of resistance takes longer than in the Southern Hemisphere, drawing us to the conclusion that resistance is likely to develop first where anthelmintics are used most frequently (Sangster *et al.*, 2002). In Australia, the prevalence of resistance to the benzimidazole and levamisole/morantel groups has increased rapidly since the discovery of benzimidazole resistance in 1968, and has continued increasing despite the introduction of regional nematode control programmemes in the mid 1980s (Waller *et al.*, 1995). There are cases where the prevalence of resistance and the cost of control failure are so high that industries are threatened (Martin, 2000; Sangster, 2001).

The cause of resistance developing is likely to be due to over-use, under-dosing, faulty dosing equipment and the continuous use of drugs within the same chemical group (Waller, 1993; Hennessy, 1997a). However, Borgsteede *et al.* (1996), stated that under-

dosing does not always promote resistance (refer to section 2.3.4.). Hennessy (2000) suggested that it was a lack of understanding of mode of drug actions, the absence of epidemiologically-based management programmes, indifference, ignorance and/or bad advice which resulted in incorrect or excessive chemical use.

On an individual farm basis, the best protection against the introduction of anthelmintic resistance is to carefully quarantine new animals until disease status is established and an effective treatment administered (Coles, 2002). However, when multiple anthelmintic resistance is already established on a farm, the eradication of resistant nematodes is difficult, particularly in warm, moist climates where free-living stages can survive for long periods of time on the pasture. When a resistance-selecting anthelmintic has been withdrawn from use for many years, there is no, or very little, reversion of resistance (Coop and Jackson, 2000; Sangster, 1999; 2001; Leathwick *et al.*, 2001; Keyyu *et al.*, 2002). Therefore, rapid re-emergence of resistance should be expected if the same drug is re-introduced (Coop and Jackson, 2000). This explains the necessity to find methods of controlling nematodes other than by chemotherapeutic methods.

1.3.4. *Growing concerns over chemical use in the food industry*

With the growing concerns over current farming methods, including the possibility of chemical residues in the food chain and the environment, it is appropriate that more sustainable approaches of controlling nematode infections be investigated (Waller, 1993; Leathwick *et al.*, 1997; Williams, 1997). It has often been assumed that these compounds only affect the desired target organisms (Gronvold *et al.*, 1996); however, few anthelmintics and endectocides currently used are considered to be completely metabolisable into inactive molecules (McKellar, 1997). This can lead to chemical residues excreted in the urine or faeces, which may affect non-target organisms (Herd *et al.*, 1996). McKellar (1997) concluded that it is the avermectins and milbemycins which are likely to exert the greatest ecotoxicological risks. For example, Herd *et al.* (1996) and Alvinerie *et*

al. (1998) found that concentrations of ivermectin potentially toxic to dung-breeding or dung-feeding invertebrates, were excreted in the dung of cows treated with an ivermectin Sustained Release Bolus, and in the dung of cows treated with the Pour-on or injectable formulations, thus harming organisms capable of biological control of nematodes (refer to section 1.4.6). Wall and Strong (1987) also found that faeces of calves fitted with ivermectin releasing rumenal boluses at 40μg day^{-1}, failed to degrade in the normal way and concluded that this failure was due to the absence of dung-degrading insects. However, with the small total number of animals on which avermectin and milbemycins are used it is unlikely that on a global or regional scale that it would have a significant ecotoxicological impact, but within a locality it may have less certain consequences (McKellar, 1997). Despite the obvious beneficial potential to reduce parasites, there may be detrimental activity on the host animal's health, and the possibility of residues accumulated in the tissues which may ultimately have adverse consequences for human health (Woolaston and Baker, 1996).

These concerns have led to a growing number of producers adopting "more sustainable" or "organic" husbandry programmes, which look to decrease or eliminate the application of man-made chemicals to their herds/flocks (Cabaret *et al.*, 2002). However, a major problem in the conversion of small ruminant production from conventional to organic is parasitic disease control and prevention, along with grazing management and organic feed provision (Ronchi and Nardone, 2003). Helminth infection is usually, but not always, more intense on organic farms than on comparable conventional farms and the diversity of infection (more species and in balanced proportions) was always higher in organic farms (Cabaret *et al.*, 2002). For organic producers, gastro-intestinal nematodes are arguably the most economically important disease causing organisms affecting their management systems and for those who don't want to use chemical anthelmintics, control options are few (Gasbarre *et al.*, 2001). Non-chemotherapeutic control options therefore need to be researched and field evaluated as a matter of urgency (Waller and Larsen, 1996).

1.3.5. *Sustaining the efficacy of the modern anthelmintics*

With resistance in mind it is important to look at sustaining the anthelmintic efficacy that is still available and therefore slowing down the occurrence of anthelmintic resistance. Intensive nematode control with anthelmintics to maximise production is simply not sustainable any more (Leathwick *et al.*, 2001) and the eradication of gastrointestinal parasites seems impossible (Roos, 1997).

However the understanding of how anthelmintics work is still incomplete, and there is only a meagre grasp of the pharmacodynamic principles that underlie their efficacy, and what is more worrying, the ability to discover novel anthelmintics is waning (Geary *et al.*, 1999). It is this lack of interest in antiparasitic drug resistance from all the bodies which support veterinary research that is a major reason for the shortage of information on both the epidemiology and the optimal management of anthelmintic resistant nematodes (Coles, 2002). With significant breakthroughs in the discovery of new classes of broad spectrum anti-parasitic drugs occurring less frequently than once per decade over the last 40 years (Pritchard and Tait, 2001), and little likelihood of a new broad-spectrum compound being available in the near future (Hennessy, 1997a; Waller, 1999), a non-chemical means of nematode control is a necessity for the future control of nematodes in most species grazing farm animal. However it is the difficulties of devising and introducing non-chemical means of parasite control without reducing productivity that has contributed to the over dependence on drugs making resistance even more difficult to conquer (Sangster, 2001).

"Although research into non-chemotherapeutic parasite control alternatives, such as host genetic resistance, grazing management, worm vaccines and biological control continues, collectively they are unlikely to dispense with the need for timely intervention of effective anthelmintic treatment" (Waller, 1997). Research priorities must look to improve the monitoring of resistance; the formulation and application of techniques to slow the development of resistance and hence sustain/maximise the useful lifespan of present and

any future anthelmintic compounds (Barrett *et al.*, 1998). More interest in the molecular basis for anthelmintic resistance needs to be found as it remains largely obscure to date (Geary *et al.*, 1999).

Integrated pest management (IPM), through utilising good husbandry and anthelmintics has been suggested as the only way which sustainable parasite control of livestock can be achieved, using anthelmintics in support of other control methods, rather than substituting them (Barger, 1999; Githigia *et al.*, 2001). For example, simple measures such as slowing digesta flow rate by removing feed from sheep for 24 hours prior to drenching, can prolong the availability of drug at sites of absorption by host and parasite (Hennessy, 1997a). A possibility would be an IPM system which would work by improving host resistance using non-chemical means to control parasites, using chemicals wisely, improving monitoring of infection and resistance and understanding the host-parasite relationship. Therefore some level of parasitism and some production loss may have to be tolerated (Waller, 1993, Sangster, 2001).

Only where control technologies require no anthelmintic treatment at all, or where there are literally no survivors of anthelmintic treatment, can it be confidently predicted that there will be no selection for resistance (Barger, 1997). Barnes *et al.* (1995) suggested that highly effective anthelmintics, or combinations of anthelmintics, because they have very few survivors, select less strongly for resistance than less effective drugs. However later works/reviews by Sangster (1999) and Coles (2002) suggested that selection for resistance occurs most readily in the range of efficacy 90-99.99%. Therefore avoiding efficacy in this range could be beneficial, by only treating some animals on a farm with anthelmintics or/and by ensuring exposure of animals to anthelmintic susceptible nematodes or treating to achieve reduced efficacy (<90%), could be steps to reduce selection pressure and delay the development of resistance. Barnes *et al.* (1995) determined from work on a mathematical model that gross under-dosing delays resistance slightly without impairing worm control, suggesting that a more practical option would be to not dose 20% of the

animals. This in turn could delay resistance by about five years. Coles (2002) even suggested that just the sign of scouring may be a good enough indicator of a need to treat and those that do not appear to require treatment should be left untreated. However, scouring in an individual sheep does not necessarily indicate a high worm burden in that animal (Colditz *et al.*, 1996). The traditional advice that under-dosing always promotes resistance may be wrong (Coles, 2002). However it is difficult to exploit the understanding of the qualitative relationship between dose level and the risks of promoting anthelmintic resistance to manage resistance, because the values of the relevant parameters (such as resistance allele frequency at the treatment start and parasite fecundity and survival in absence of the drug) are almost completely unknown in each case (Borgsteede *et al.*, 1996).

Reports of drug resistance in cattle have been few and far between, but some recent investigations are beginning to provide evidence of its existence (Williams, 1997). The much greater prevalence of resistant nematodes in sheep, when compared to cattle, may be explained by cattle being treated only in their first grazing season, and then often grazed on land where the mature non-treated animals have grazed, thus exposing them to susceptible larvae. On the other hand adult sheep are treated as well as the young, increasing the development of resistance (Waller, 1993; Coles, 2002).

1.4. Alternative control measures

1.4.1. *The likelihood of ethnoveterinary (herbal) treatments as anthelmintics*

Hammond *et al.* (1997) reviewed the current uses of anthelmintic plants in tropical veterinary medicine. Anthelmintic plants offer a traditional alternative to manufactured anthelmintics that is both sustainable and environmentally acceptable. Such plants could have a more important role in the future control of helminth infections (Hammond *et al.*, 1997). Many anthelmintic compounds, with different modes of action have been isolated from plants and these could be of value where resistance has developed to the

manufactured anthelmintics. However in contrast to human medicine, 'phytotherapy' is not well developed in veterinary medicine and still lacks general acceptance. There is no doubt that some plants contain active principles that have an anthelmintic effect, however, comprehensive data on dose levels and methods of use are usually lacking (Hammond *et al.*, 1997). Some of the plants of interest against GINs of sheep would be *Heracleum* spp., (Gadzhiev and Eminov, 1986 cited in Hammond *et al.*, 1997) *Hedysarum coronarium* (sulla or Grassland Aokau) which contains condensed tannins (Niezen *et al.*, 1995).

Sheep fed forages containing condensed tannins have been reported to have lower GIN infections compared to those fed tannin free forages (Niezen *et al.*, 1995; 1998a; 1998b; 2002). The anthelmintic effects of condensed tannins had been attributed to an improved protein supply to the lower intestinal tract, (protected protein possibly improves resistance to parastic infections (Coop and Kyriazakis, 1999; Donaldson *et al.*, 1998; 2001), although (Butter *et al.*, 2001) suggested that dietary *Quebracho* tannin may reduce GIN burdens through a toxic effect that requires direct contact between parasite and tannins. Athanasiadou *et al.* (2001b;a) found that administering condensed tannin extract (*Quebracho*) in the feed of sheep infected with either intestinal nematodes or abomasal nematodes reduced FECs in the intestinal nematode infected sheep when compared to untreated controls but did not reduce the FECs of the sheep infected with abomasal nematodes. However investigations by Athanasiadou *et al.* (2001a) did not observe the previously seen anthelmintic properties of condensed tannins following *ad libitum* intake of either low- or high- protein foods supplemented with *Quebracho* extract leading them to the conclusion that higher levels of *Quebracho* may need to be fed when the animals are fed *ad libitum*.

1.4.2. *The use of good pasture management to reduce the dependency on anthelmintics*

Malan *et al.* (1997), reported that animals confined by fences, even those surrounding vast areas such as national parks can become overpopulated and cause an increase in parasitic disease. Excessively high stocking rates usually go hand in hand with a deterioration of pasture quality and quantity, and thus an increased incidence of parasitism and disease. Domestication and intensification have tipped the balance in favour of parasites by providing an abundance of susceptible hosts and favourable pasture micro-environments for the free-living stages (Waller and Faedo, 1996).The mobility lost in sedentary systems should therefore be replaced by sound pasture and animal management to manage the risk of any disease including parasitic disease (Schillhorn van Veen, 1997). However making pasture management succeed in reducing nematode infections requires a comprehensive knowledge of the parasite epidemiology as it interacts with the host in a specific climatic, management and production environment, without this knowledge, anthelmintic treatment is either given suppressively, which helps promote anthelmintic resistance, or therapeutically, which can risk clinical disease and production losses (Barger, 1999).

Management as a method of nematode control is more a method of avoiding nematodes rather than controlling them as integrated grazing systems are designed to minimise larval ingestion (Niezen *et al.*, 1996). Githigia *et al.* (2001) compared the 'dose and move' and 'move' only strategies of grazing management. They showed similar production parameters in both systems. Consequently, by weaning lambs at the beginning of July and moving them from an infected pasture to a clean pasture with abundant high quality grass will help prevent gastro-enteritis and achieve good production whether the move is accompanied by anthelmintic treatment or not. Nomadic and transhumant movement, as practiced in Africa, is the ultimate rotational grazing system, probably aimed more at nutritional security than disease prevention. However, it could be questioned whether such a system can contribute significantly to parasite control, especially if regular annual patterns are followed, because parasites are able to adapt rather efficiently (Schillhorn van

Veen, 1997). In the wet tropics a grazing system of 10 paddocks grazed for 3.5 days at a time could reduce the frequency of anthelmintic treatment to once per year (Gill and Le Jambre, 1996). Any pasture management needs to bear in mind that although young animals need protection from pathogenic worm burdens they also need exposure to nematodes to acquire and maintain immunity (Barnes *et al.*, 1995; Colditz *et al.*, 1996; Vercruysse and Claerebout, 2001), and with the eradication of nematodes being improbable, and in most cases, not feasible, the animals would have to become exposed sooner or later.

A second measure to prevent exposure to parasites involve putting nematode-free animals onto a 'clean pasture' (a pasture not grazed by the same host species for a year or more), after these animals have been treated with anthelmintic (Barger, 1997). This 'dose and move' strategy can suppress egg output for several months rather than the average 3-4 weeks (Barger, 1997). This method has been advocated as an option for practical control in the UK (Coles and Roush, 1992), and combines a move of ewes with lambs or weaned lambs to clean pasture in midsummer with an anthelmintic treatment just before the larvae from the eggs shed on the original pasture appear at dangerous levels (Michel, 1976, Githigia *et al.,* 2001). However the dose and move strategy which works well in the reducing the loss of productivity due to larval challenge is almost designed to select for anthelmintic resistance due to contamination of the field originating only from resistant worms which have survived the treatment (Barger, 1996; Coles, 2002). Indeed any given number of anthelmintic treatments which reduce the size of the larval population especially with movement to "clean" pasture rather than "dirty" has the potential to increase selection pressure for anthelmintic resistance (Niezen *et al.*, 1996). Under the warm, moist conditions of the temperate regions a strategy for reducing this possibility of encouraging anthelmintic resistance could be a carefully monitored introduction of anthelmintic susceptible worms to the animals when moving them on to clean grazing.

A third method can 'dilute' the herbage infestation by concurrently grazing two species of animal or same species with different susceptibilities, with a greater number of the helminthologically inert animals than susceptible (Schillhorn van Veen, 1997). The advantages of mixed and alternate grazing systems (especially for non-related species such as cattle, horses or in some African steppe systems, wildlife) on reducing nematode loads are well known (Schillhorn van Veen, 1997). A multiple host-species situation provides the benefits of predation and scavenging, a better 'vacuum cleaner' effect of each other's parasites (Malan *et al.*, 1997). Care needs to be taken if pastures are infected with *Trichostrongylus axei* and *Haemonchus placei* which can affect different host species so could cause a problem when pasture are alternately grazed by different species or mixed grazed, goats and sheep should not be used as their parasite species are overwhelmingly shared (Barger, 1996).

Even though grazing management is useful especially when anthelmintic treatment is incorporated, an anthelmintic-free system would be more likely to succeed in the tropics where the life of the free-living stages is relatively short (6-8weeks) than in temperate areas where larvae can survive for months (Barger, 1996). In hot dry climates moving to clean grazing may not even be necessary due to the free-living stages being more easily destroyed (Coles, 2002). Barnes *et al.* (1995) suggests that without grazing management, the heavy reliance on drugs rapidly leads to very high levels of anthelmintic resistance. However grazing management must be practicable and achieve levels of parasite control sufficient to meet realistic production objectives (Niezen *et al.*, 1996).

In general, overstocking can cause animals to graze in a pattern they may not do if in a less intensive management system. Animals generally avoid eating around their own faeces and often wouldn't eat grass lower than a couple of centimetres off the ground (Stromberg, 1997). By avoiding overstocking and therefore keeping the sward tall enough provides the animal with adequate forage and better nutrition which can lead to better defence against parasites. This would also help avoid the majority of nematode larvae which do not venture

far from the faeces (normally no more than 30 cm, with optimal larval recovery being approximately 5 cm from the faecal pat and a couple of centimetres from ground level (Wells, 1999)). This may explain why sheep have more nematodes problems as they graze closer to the ground than cattle, and find it more difficult to avoid their faeces, exposing them to more larvae (Wells, 1999).

1.4.3. *Exploiting host genetic resistance against nematodes*

In large ecosystems free from human intervention, parasites and predators fulfil an important role in the selection of host populations for fitness (Malan *et al.*, 1997). Young animals are often subject to large parasite infections, causing weaker animals and those with neither innate resistance nor the ability to mount an effective immune response, to succumb to predators before they can contribute to the gene pool (Malan *et al.*, 1997). Farming has developed an environment where natural 'survival of the fittest' selection pressures are minimal, because of protection from predators and chemotherapy and enables the ability to select for desired properties such as high milk production and fast weight gain. However these may be selected at the expense of natural resistance against nematodes and other diseases. Sheep breeds that have never been treated by drugs have a much higher level of natural resistance to parasites than the breeds selected for high production and that have been regularly treated (Barger, 1989). Malan *et al.* (1997) suggested that, in the face of the increasing anthelmintic resistance, the farmer should take over the role of predator in selecting for fitness. In Africa, where disease pressure has been particularly high, selection for breed resistance (either on purpose or by default) has been a necessity to enable livestock production (Schillhorn van Veen, 1997). The most commonly quoted example of natural disease resistance is the trypanotolerance of the humpless dwarf cattle breeds of the West African forests, such as the Ndama, Mayumbe, Muturu and Baloué (Tizard, 2000), whilst European breeds, such as the Friesian, cannot survive in these areas. This suggests that genetically selecting for animals with natural resistance

against nematodes is possible, but it may be at the expense of traits desired by producers. Indeed, Gasbarre and Miller (2000) suggested that the usefulness of resistant breeds will depend on the commercial requirements of the producer. Breeds in the past have been selected for particular production systems and environments, and can rarely be changed without some production penalty and in some cases there may not be a realistic alternative (Barger, 1996).

Research in the last 20 years has firmly established that it is possible to exploit genetic variation in resistance to nematode parasites of sheep by genetic selection. Certain breeds show an increased resistance to nematodes, for example, Merinos, Red Maasai, Louisiana Native and Scottish Black Face sheep, when compared to others, show a lesser faecal egg output, and therefore reduce pasture contamination (Gray, 1997). There is also variation in nematode burdens/egg output between individual sheep in a flock, with a minority of individuals, usually harbouring the majority of the nematodes (Gasbarre and Miller, 2000; Tizard, 2000) and the same over-dispersed distribution of nematodes occurs in humans with the possibility of 20% of the population harbouring 80% of the nematodes (Lunn and Northrop-Clewes, 1993). Stear *et al.* (1997) and Gasbarre and Miller (2000) found no influence of host genetics on nematode numbers; it was the effect on worm size and fecundity rather than on worm burden that were the heritable traits.

There are two possibilities for selection, either breeding for resistance by selecting animals with low FECs, or breeding for resilience by selecting animals which still perform well when infected (Woolaston and Baker, 1996). Whilst nematodes have a better chance to adapt to resistant hosts there are far more disadvantages to breeding for resilience than for resistance, some of which include the risk of disease requiring good vigilance, lower productivity and no epidemiological benefits as the stock may harbour many nematodes (Woolaston & Baker, 1996). Indeed FEC has proven to be an effective selection criterion for improving resistance and is simple to measure. It is also better than other methods, for example, circulating eosinophil counting (Woolaston *et al.*, 1996). Khan *et al.* (1999)

concluded that breeding for resistance to nematode parasites, by selection on the basis of FECs at 4-5 months of age, is effective in reducing both FECs and the magnitude of the PPR in the periparturient ewe.

Gray (1997), however, suggested that breeding alone is not a viable option for worm control and looked at the need to investigate combining genetic selection with either vaccines or nutritional supplementation. Cabaret (2003) evaluated the use of rams selected for resistance but found their influence on production was ambiguous, although the heritability of resistance to nematodes was relatively high. The time scale in Cabaret's experiment was probably not long enough for the selection process to produce any clear advantage. If breeding alone was a viable option for nematode control, even if a scheme was implemented immediately it would require a further 10 years of selection to achieve substantial reductions in the effects of nematodes on production (Gill and Le Jambre, 1996).

1.4.4. *Nematode vaccines*

In the face of anthelmintic resistance vaccination against nematodes would appear to be the control method of choice. If one could be developed, current concerns about tissue residues and ecotoxicity in the environment increase its appeal (Klei, 1997). There is a great deal of evidence that animals, in time, develop substantial resistance against reinfection with parasites; this should challenge the skills of scientists to produce vaccines to induce acquired immunity (Emery, 1996). Vaccination should be possible against parasites where acquired immunity is evident, unlike those parasites that do not elicit any form of immune response, for example, fasioliasis (Dalton and Mulcahy, 2001). There is a radiation-attenuated larval vaccine against the lungworm, *Dictyocaulus viviparus,* in cattle, which has been used commercially for over thirty years (Martin, 2000; Tizard, 2000). With the exception of the vaccine against *D. viviparus* (Martin, 2000), a recombinant vaccine against the tick, *Boophilus microplus* (Willadsen *et al.*, 1995), and the cestode, *Teania ovis*

(Rickard *et al.*, 1995) there has been little evidence of any other parasite vaccines being close to coming onto the market (Klei, 1997). Unfortunately though, attempts to produce vaccines for gastrointestinal nematodes of livestock using the same attenuation procedures, and the molecular approach using antigenic parasitic material, have failed to provide a viable commercial product, to date (Waller, 1997; Pritchard and Tait, 2001). It is perhaps both the complexity of the nematodes and their distinct evasion mechanisms which have made vaccination using whole killed parasites impossible (Meeusen, 1996). Meeusen (1996) and Smith (1999) surmised that the main stumbling blocks that have so far precluded the development of effective anti-nematode vaccines are the identification and isolation of the protective parasite antigens, and the induction of the appropriate protective immune effector mechanisms through vaccination. The ideal vaccine would need to have an efficacy good enough to reduce parasites to lower than numbers that cause significant production losses, have a broad-spectrum anthelmintic activity and be safe, neither producing adverse reactions themselves or when the host is exposed to nematodes (Klei, 1997).

There is evidence of effective vaccination against *Haemonchus contortus* in sheep, Kabagambe *et al.* (2000) found that *H. contortus* gut antigens formulated into a vaccine offered some benefit in reducing pasture contamination. There are many molecules with protective properties against *H. contortus* and this may have potential for the future development of a molecular vaccine against this parasite (Dalton and Mulcahy, 2001). Most vaccines studied have been aimed at *H. contortus* hidden gut antigens (Knox and Smith, 2001a), which have been effective against this blood-sucking parasite and have showed good results even in lambs and periparturient, females, however immunity is not boosted by this vaccine so it would be thought that regular boosters may be needed (Smith, 1999). This may not be so as this vaccine effects the older, blood drinking parasites and larvae can still survive and promote a natural immune response (Smith, 1999). However,

the relatively low market share for this parasite alone may not support its production commercially (Dalton and Mulcahy, 2001).

Dalton and Mulcahy (2001) suggested that what was needed was a broad-spectrum vaccine against *H. contortus, T. circumcincta* and *Trichostrongylus* spp. that would be able to compete with the broad-spectrum anthelmintics on the market. Attempts at finding a vaccine against the two non-blood-sucking nematodes have met with positive, but much less consistent, results (Dalton and Mulcahy, 2001). There is a need to find a cost-effective means of antigen delivery to mucosal surfaces that can induce immune and reinforce protective naturally acquired immune responses (Emery, 1996). Optimistically Smith (1999) predicted that within the next decade there would be the launch of the first antigen nematode vaccine for sheep.

1.4.5. *Returning to copper therapy as an anthelmintic treatment*

Waller (1999) discussed the reversion back to treatments involving copper, not unlike the drugs used prior to 1930 although not as hazardous as the concoctions of copper, arsenic and nicotine. The use of copper-oxide wire particles (COWP) have been studied with positive results against the abomasal dwelling nematodes by Bang *et al.*, (1990). Waller (1999) stated that COWP should be investigated further because of their high efficacy, prolonged activity, cheapness and relative safety as they are a form of ultra-low-dose copper administration against *H. contortus*. Indeed recent studies have shown that 2 to 6g COWP can significantly reduce *H. contortus* faecal egg output (Knox, 2002; Burke *et al.*, 2004).

1.4.6. *Biological control of nematodes*

Biological control is the use of natural enemies which maintain host population at levels lower than would occur in the absence of enemies (Waller & Faedo, 1996). Gronvold *et al.* (1996) and Waller and Faedo (1996) described many organisms capable of harming

nematodes, such as nematopathogenic bacteria, amoeba and the effect of earthworms digesting faeces, but it is highly unlikely that they will offer opportunities for biological control of nematode parasites of livestock. However the nematopathogenic organism of most interest to the veterinary profession would be the nematopathogenic fungi.

Larsen (1999), has described prospects for controlling animal parasitic nematodes by the nematopathogenic micro-fungi – *Duddingtonia flagrans*. This fungus can be administered as spores in the feed of the host, as it has been found naturally in sheep, cattle and horse faeces (Larsen, 1999). This fungus develops in the expelled faeces and traps the nematodes in 'noose like' bindings from which the fungus inserts hyphae into the victim and digests it (Gronvold *et al.*, 1996; Larsen, 1999). Other nematopathogenic fungi are the endoparasitic fungi (*Harposporium helicoides*) which infect nematodes by spores which are ingested which develop and absorb the body contents (Gronvold *et al.*, 1996). Waghorn *et al.* (2002) found evidence that both *H. helicoides* and *D. flagrans* could control *T. circumcincta* in field trials. The formulation of effective preparations was the major limiting factor to commercial development of nematopathogenic fungi (Martin, 2000). Waller *et al.,* (2001; 2001a) reported promising results with both administering feed supporting the nematophagous fungus and prototype control release devices (CRDs) containing the chlamydospores of *D. flagrans* against nematode larvae in the faecal pat.

Other organisms of interest are the dung beetles (Scarabaeidae) (tropical and sub-tropical areas) and earthworms (Lumbricidae) (cultivated land in colder regions) (Gronvold *et al*, 1996). These organisms have been shown to reduce nematodes by breaking up pats, increasing drying-up and burying the faeces in turn increasing nematode mortality (Gronvold *et al.*, 1996; Waller and Faedo, 1996). Unfortunately both species are reliant on favourable weather conditions to perform, earthworms need cool, moist conditions and dung beetle prefer warm, dry conditions (Waller and Faedo, 1996).

However there are worries about introducing a natural enemy due to its possible effects on non-target organisms (Waller and Faedo, 1996). There are examples of biological control

such as myxomatosis and rabbits (Australia) offering outstanding control, the disease felled 95% of the rabbit population (Barger, 1996), but also examples of it going wrong such as the Cane toad against cane beetles (Australia) and the Euglandina against Achatine snails (Tahitian Islands) (Waller and Faedo, 1996). Now even the myxomatosis success story is threatened by resistant rabbits and a less virulent strain of myxomatosis (Barger, 1996). Very rigorous assessment of the environmental impact would have to be performed before field release of any biological control (Waller and Faedo, 1996).

1.5. The periparturient rise in faecal egg counts

Outbreaks of parasitic disease in domestic ruminants are generally confined to young animals, particularly in the period from weaning to one or two years of age (Barger, 1993b; Colditz *et al.*, 1996; Khan *et al.*, 1999). The greater susceptibility of young animals is ascribed to the defective development of protective acquired immune responses to worm infection compared with adults (Watson and Gill, 1991a). Adult animals express acquired immunity to gastrointestinal nematodes (GINs) due to continuous exposure to parasites under normal grazing management (Brunsdon, 1982). This is usually highly effective; the majority of incoming infective larvae are rapidly rejected or their development is retarded (Houdijk *et al.*, 2001b). Thus adult ewes usually harbour a small number of adult nematodes whilst the number of eggs in their faeces is often low or zero (Gibbs, 1986). Crofton (1954), however, noted that there was an increase in faecal egg counts that was directly related to the time of lambing.

Lloyd (1983) reported evidence that pregnancy promotes immunosuppression to help prevent rejection of the foetus which is a uniquely successful natural allograft; this suppression is not specific to the foetus and extends to a variety of infections including bacteria, viruses, protozoa and helminths. There are reports of reduced immunity to malaria in pregnant women resulting in higher parasitaemias than age-matched, non-pregnant women (Lloyd, 1983; Menendez, 1995; Smith, 1996). Also there is abundant

evidence of ewes showing a marked increase in susceptibility to infection with nematodes in late pregnancy and during lactation (Field *et al.*, 1960, O'Sullivan and Donald, 1970, Connan, 1976, Lloyd, 1983, Jeffcoate *et al.*, 1992, Barger, 1993b, a, Khan *et al.*, 1999), with this reduced immunity being rapidly recovered after weaning (O'Sullivan and Donald, 1973; Connan, 1976; Barger, 1999). Connan (1976) and Lloyd (1983) have listed other domesticated species which have been reported to exhibit signs of the PPR including sows, cattle, dogs, mice, rats and guinea-pigs. Malan *et al.* (1997) reported that ewe impala parasite burdens peak during the lambing season. Even though there is a marked reduction in immunity to nematodes during pregnancy/lactation, during which time, clinical disease can occur, or a marked increase in FECs, the periparturient female is still, on average, more resistant than previously unimmunised animals (Lloyd, 1983).

The parasitized periparturient ewes carry more adult nematodes (Donald *et al.*, 1982; Smith *et al.*, 1983) and a rise in faecal nematode egg counts (Field *et al.*, 1960; Gibbs, 1986; Donaldson *et al.*, 1998; Tizard, 2000) than parasitized barren ewes. Around parturition, there is an increased establishment of incoming larvae, a decreased ability of the ewe to suppress egg production by the female worms and to expel developed worms (O'Sullivan and Donald, 1970; Lloyd, 1983; Gibbs, 1986; Kahn *et al.*, 1999). The phenomenon has often been associated with lactation rather than pregnancy (Connan, 1968; O'Sullivan and Donald, 1970; Brunsdon, 1970; Brunsdon and Vlassoff, 1971; Leyva *et al.*, 1982), although others report the phenomenon commencing in late pregnancy (O'Sullivan and Donald, 1973; Reid and Armour, 1975).

1.5.1. *The consequences of the periparturient rise in faecal egg counts*

Increased susceptibility of the periparturient ewe to nematodes can ensure transmission to the next generation of animals (Lloyd, 1983). Under temperate conditions, the ewe plays an important role in the epidemiology of GIN parasitism because the larvae derived from the periparturient rise in faecal egg count, together with those that have survived over

winter on the pasture, are the major sources of gastrointestinal nematode infection for young lambs (Heath and Michel, 1969; Connan, 1976; Gibbs, 1986; Jackson *et al.*, 1988). (Figure 1.3). Parasitised periparturient ewes have lower bodyweight after lambing, produce less milk and wool and lose more bodyweight during lactation (Thomas and Ali, 1983) than non-infected periparturient ewes.

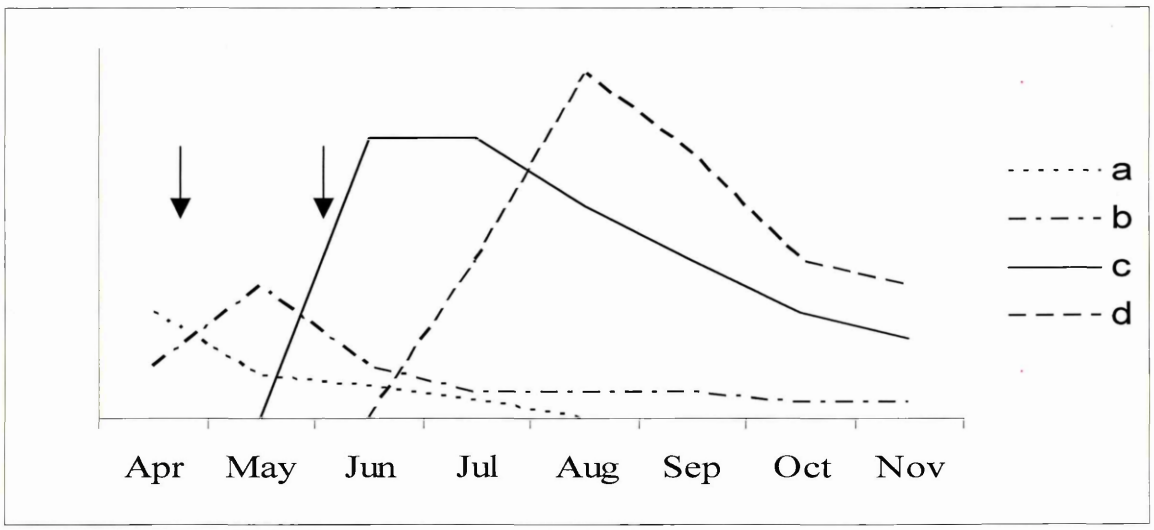


Figure 1.3. A schematic illustration of the seasonal dynamics of pasture infectivity for parasitic gastroenteritis of small ruminants in temperate areas (Adapted from Kassai, 1999). (The first arrow = parturition, the second = weaning)

a- Infective larvae which survived the winter
b- Ewe faecal egg output (PPR)
c- Lamb faecal egg output
d- Infective larvae on the pasture

Overcoming this breakdown in the expression of immunity may decrease intake of infective larvae by lambs and thus potentially increase their growth and performance and this may lower the dependency on chemoprophylaxis as a means of GIN control in the lambs (Houdijk *et al.*, 2001c).

1.5.2. *The possible causes of the PPR*

Various hypotheses have been put forward for the occurrence of the PPR including hormonal effects, seasonal effects, lack of antigenic stimulation, stress and nutrition, but the phenomenon remains poorly understood (Lloyd, 1983; Barger, 1993; Kahn, 1999;

Houdijk *et al.*, 2001c). It is evidently a multifactorial phenomenon, the mechanism of which needs clarification (Coop *et al.*, 1990).

1.5.3. *The immunology of the pregnant female*

During pregnancy, modifications occur in virtually every facet of the immune response (Luppi, 2003). Lloyd (1983) concluded that immunosuppression occurs during pregnancy and lactation and that it is primarily the T-cell dependant immune responses which are affected. Helper T lymphocytes ($CD4^+$) produce cytokines involved in promoting the immune cells against certain infections. There are two subsets of helper T cells; Th-1 and Th-2, the former of which is largely involved in the expulsion of intracellular parasites particularly protozoa and Th-2 cells produce the cytokines – interleukin 4 (IL-4), IL-5 and IL-10 amongst others. These are involved in the promotion of immunoglobulin E (IgE) antibodies and eosinophils, which are largely involved with the expulsion of extra cellular parasites such as the GINs (Gasbarre, 1997; Roitt *et al.*, 1998; Gasbarre *et al.*, 2001). Pregnancy has been associated with a significant decrease in the frequency of T helper cells, in particular the Th-1 cells to help protect the foetus from destruction (Luppi, 2003). This could explain the increases in protozoal diseases during pregnancy such as malaria (Menendez, 1995; Smith, 1996), *Neospora caninum* (Quinn *et al.*, 2002) and *Leishmania major* (Piccinni *et al.*, 2000, Hunter and Reiner, 2000) but cannot explain the reduction in immunity against GINs as there is a presence of a Th-2 immune response bias (Luppi, 2003). Indeed, mice which mount a Th-1 response against nematodes become heavily and chronically infected unlike those which mount a predominantly Th-2 response (Tizard, 2000). More evidence of the immunological changes consistent with a weakening in the Th-1 responses during pregnancy include a temporary remission of symptoms of the T-cell-mediated autoimmune diseases - rheumatoid arthritis and multiple sclerosis (Piccinni *et al.*, 2000; Luppi, 2003). In pregnancy, the immediate innate immunity, such as granulocyte and monocytes involved in the expulsion of bacteria, are usually increased

where the adaptive immunity, especially the cells promoted by the Th-1 sub-set are suppressed creating a balance and helping maintain the mother's defences against some infections whilst helping protect the foetus from destruction (Luppi, 2003).

Huntley *et al.*, (2004) noted that there was a reduction in mast cells and globule leukocytes during the PPR when protein supply was restricted, these cells are involved in the response against nematodes. As immunosuppression appears to occur both during pregnancy and lactation in most species, therefore an immunosuppressive factor, not yet identified, involved in pregnancy and lactation may be the real source of interest (Jeffcoate *et al.*, 1992). Jeffcoate *et al.* (1992) found that the rise in faecal egg count was synchronous with a rise in the anti-helminth Immunoglobulin A (IgA) levels in the plasma. The rise in IgA levels was attributed to lactation as increased levels of IgA are transported to the milk, possibly at the expense of IgA levels in the gut, despite IgA being almost totally synthesised in the gastrointestinal mucosa. The main immunological mechanism that regulates worm length and fecundity of *T. circumcincta* is the quantity of parasite-specific IgA (Stear *et al.*, 1997).

1.5.4. *The effects of endocrine status on the PPR*

O'Sullivan and Donald (1970) and Connan (1976) assumed that the reduction in immunity was primarily of endocrinal origin. Lloyd (1983) hypothesised that a variety of hormones were involved, and Tizard (2000) also believed that the influence of age or gender on helminth populations appeared to be largely hormonal. Piccinni *et al.* (2000) showed that hormones enhanced during pregnancy can affect the development of Th-1 and Th-2 responses. During pregnancy there is a transient depression of cell-mediated (Th-1) immunity which may be explained by the increased production of certain hormones and proteins that occur to help maintain gestation (O'Sullivan and Donald, 1970).

Prolactin, the principal lactogenic hormone, was originally suspected as being of primary importance in the loss of immunity to nematodes in periparturient ewes because of the

synchronous appearance of the PPR and elevated plasma prolactin in ewes (Connan, 1976). However, increased prolactin has been shown to occur in the early stages of pregnancy in rats when increased susceptibility to nematodes is not apparent (Connan, 1976). Coop *et al.* (1990) also concluded that an increase in prolactin concentration was not the sole factor responsible for the periparturient rise in FECs. Inhibition of prolactin production has no effect on the rise in FECs (Jeffcoate *et al.*, 1990). It is mainly cortisol and oestrogens that have been associated with in vitro suppression of cell-mediated (Th-1) responses (Menendez, 1995). Cortisol (Connan, 1973) and oestradiols in high concentrations have been shown to suppress immune responses, although this was at levels a lot higher than in serum during pregnancy (Lloyd, 1983). Progesterone has also been associated with decreased immune responses and at levels similar to those found in the human placenta and plasma (Lloyd, 1983). However, Coop *et al.* (1990) found that the periparturient rise in FECs preceded the usual periparturient rise in cortisol and occurred at a time when placental progesterone levels were decreasing. Barger (1993b) suggested that hormonal suppression of immunity during pregnancy was unlikely to be a primary factor in the PPR. Another viewpoint when considering hormonal effects as the cause of the PPR could be the influence of hormones such as the adrenal glucocorticoids that are enhanced during stress. Corticosteriod administration has been shown to produce a profound decrease in circulating eosinophils (Parrillo and Fauci, 1979).

1.5.5. *The effects of mental and traumatic 'stress' on the PPR*

Malan *et al.* (1997) have found that individual animals that had a previous history of stress, such as a wound or bone fracture, would harbour a parasite burden far higher than the usual levels, with counts being up to 100 times higher than in healthy animals. Lennard and Browell (1993) found that trauma can cause a down-regulation of important cells such as T-lymphocytes, NK cells and the cytokines and receptors that control these cells, thus

rendering the host immunosuppressed. Stress could, therefore, be the cause of reduced immunity to nematodes and the cause of PPR in sheep. Crofton (1954) suggested that the 'spring-rise' (PPR) was enhanced by stress factors, such as parturition and possibly lactation. Physiological responses associated with stress result in immunosuppression and consequently may lead to increased susceptibility to pathogenic infections (Parillo and Fauci 1979). Connan (1976) suggested that the adrenal glucocorticoids could be involved in the PPR. Indeed stress associated with weaning of young lambs can contribute to delayed development of protective immune responses to certain gastrointestinal parasites (Watson and Gill, 1991b). Stress has also been suggested to go hand-in-hand with nutritional deprivation as a stressed animal may eat less and the pregnant ewe usually eats less during pregnancy and is hardly able to eat enough during lactation, putting pressure on the immune system. Physical and psychological stress has been shown to cause decreases in certain immune cell activity such as natural killer (NK) cells in barren animals (Nakamura *et al.*, 1997). However Nakamura *et al.* (1997) found that in pregnant animals stress does not cause the same immunosuppression. This suggests that pregnancy can counteract some of the immunosuppressive actions of stress and therefore there is a possibility that stress is not the cause of the PPR though this may not be the case during lactation. Barger (1993a) suggested that, like hormonal suppression of immunity discussed earlier, stress is also unlikely to be a primary factor in the PPR.

1.5.6. *Seasonal effects and the effects of hypobiosed larvae on the PPR*

Previously, the PPR was associated with spring; Brunsdon (1966; 1970) discussed the 'spring-rise' phenomenon. However, Connan (1976) reported that the PPR occurs in periparturient ewes independent of the season. Field *et al.*, (1960) and Gibbs (1986) attributed the rise in FECs in periparturient ewes to the maturation of hypobiosed larvae, (hypobiosis is the term describing the arrest of development at the L_4 stage for a period of time when the environmental conditions are unfavourable for the transmission of the free-

living stages (Gordon, 1970). Field *et al.* (1960) came to the conclusion that it was maturation of hypobiosed larvae that was the cause of the rise in FECs because of the fact that the housed periparturient ewes still had significant rises in FECs despite no further exposure to nematodes. O'Sullivan and Donald (1970), however, reported that there was very little evidence that there was a direct relationship between the two phenomena, and that hypobiosed larva are not essential for there to be a rise in FECs.

It appears that parasites have synchronised their reproductive cycle with that of their host's (Tizard, 2000). Larvae have hypobiosed development to synchronise its life cycle with the season best suited for its pre-parasitic development (Gibbs, 1976). When maturation of the larvae coincides with the host's PPR this provides an enhanced opportunity for the parasite's development, thus synchronising further the parasite's life cycle with that of the host (Connan, 1976)

1.5.7. *The effects of nutrition on the PPR*

Over half a century ago, Whitlock *et al.* (1943) quoted that parasitism was fundamentally a 'nutritional disturbance' and improving nutrition has indeed led to reduced production losses and mortality rates due to gastrointestinal nematodes (Waller, 1999). According to Brunsdon (1982), animals which are subject to malnutrition are less able to develop and maintain a level of resistance sufficient to protect themselves from serious parasite infection. Under conditions of marginal nutrition such as hill farms, low levels of parasitism may have a greater effect on production than under high levels of nutrition (Niezen *et al.*, 1996). Malan *et al.* (1997) reported that in natural drought situations in Africa nutritional stress is severe and the immune status of hosts compromised, leading to temporary local overpopulation and acceleration in parasite and disease transmission. The animals develop immunity to reinfection after either rain or a certain number of individuals have perished leaving less competition for food. Indeed, the periparturient rise in FECs

coincides with an increase in the nutritional requirements of the ewe due to the demands of pregnancy and lactation (Kahn *et al.*, 1999).

Lymphoid tissues are metabolically active tissues and have a high rate of cell proliferation and there is a rapid turnover of cell products and proteins, making the immune system extremely vulnerable to the damaging effects of undernutrition (Chandra, 1993, Burkholder and Swecker, 1990). Around the periparturient period, the physiological processes that underlie reproduction inflict substantial metabolisable protein demands on the ewe (Houdijk *et al.*, 2001a).

In the past ten years, interest has increased in the interactions between the nutritional status of the host and parasite establishment/rejection/fecundity, with an abundance of reviews on the subject (Barger, 1993b, Coop and Holmes, 1996, Coop and Kyriazakis, 1999). Many researchers have already studied the effects of nutrients, especially proteins, on nematode infections in lambs (Abbott *et al.*, 1985; 1988; Coop *et al.*, 1995; van Houtert *et al.*, 1995), in periparturient ewes (Donaldson *et al.*, 1998; 2001; Houdijk *et al.*, 2000a; 2001a) and sheep in general (Abbott *et al.*, 1988; Abbott and Holmes, 1990; Coop and Holmes, 1996; Knox and Steel, 1996; 1999; van Houtert and Sykes, 1996; Coop and Kyriazakis, 1999). Many immunological products such as immunoglobulins and lymphokines are proteins (Burkholder and Sweckler, 1990). Knox and Steel (1996) concluded that even just urea supplementation of sheep on low quality fibrous diets reduced infection levels and the debilitating effects of nematodes on the host. Donaldson *et al.* (1998) found that fish meal protein rather than energy supply resulted in a reduction in nematode burden. The energy requirement of the average 'unparasitised' ewe increases 2 fold (MJ day^{-1}) *pre-partum*, and a 2.9 fold increase in energy day^{-1} during lactation. Compared to the increase in requirements of digestible protein *pre-partum* of 2.6 fold and during the latter stages of pregnancy and a 5.4 fold increase during lactation, it is apparent that a ewe may become lacking in protein supply (Kahn *et al.*, 1999). Burkholder and Swecker (1990) suggest that caloric restriction may benefit and improve the immune system and may delay the aging of

the immune system and the reverse being true of over caloric supplied subjects which are suffering obesity.

A practical means of increasing digestible protein supply relative to energy supply is to supplement the animals during late pregnancy and lactation with a protein source resistant to rumen fermentation as the digestible protein (DP) to metabolisable energy (ME) requirement in late pregnancy exceeds that able to be provided by the rumen (Kahn *et al.*, 1999).

Huntley *et al.* (2004) confirmed that the PPR is observed during late pregnancy and lactation and was exacerbated by protein undernutrition. Any changes in immune responses can occur early in the course of nutritional deficiency (Chandra, 1993). Interactions between the host and nutrition can be considered from two interrelated perspectives. Firstly, the effects of nutrition on the metabolic disturbances and the pathophysiology induced by parasitism and secondly, the influence of nutrient availability of the host to mount an effective response against parasite establishment and/or development and to influence the 'resilience' and 'resistance' of the host to parasitic infection (Coop and Kyriazakis, 1999). Resilience can be considered as the host's ability to maintain a reasonable level of productivity in the face of a parasitic challenge (Albers *et al.*, 1987), and resistance is a measure of the host's ability to limit the establishment, growth rate, fecundity and/or persistence of a parasite population (Coop and Kyriazakis, 1999).

Gastrointestinal nematodes (GINs) generally reduce the nutrient availability to the host through both reductions in voluntary feed intake and/or reductions in the efficiency of absorbed nutrients (Dynes *et al.*, 1998). Therefore, the host can be seen as having a problem allocating the scarce resources among its body functions. Such body functions include not only the usual ones such as maintenance, growth and reproduction, but also additional functions that are the direct consequence of parasitism. For example, the metabolic drain caused by increasing endogenous loss of protein or tissue damage and the

ability to immunoregulate the parasite population (Coop and Kyriazakis, 1999). The expression of immunity to GINs has to compete with other bodily functions when nutrient resources are scarce.

Animals will invariably give priority, when nutrients are scarce, to nutrient allocation to the maintenance of the body protein, since this guarantees the individual's survival in the short term (Coop and Kyriazakis, 1999). It is proposed that growth and reproduction, which will ensure the survival of the species in the long term, be given the second highest priority. This implies that functions such as expression of immunity to GINs and increase of maternal bodyweight have a relatively low priority for the allocation nutrients (Coop and Kyriazakis, 1999). The result of this repartitioning of nutrients, particularly protein, is a reduction in soft tissue deposition, skeletal growth, wool and milk production (Poppi *et al.*, 1986, Holmes, 1993, Sykes, 1994). In the parasitised ruminant nutrients and protein synthesis are diverted away from production processes such as skeletal growth and muscle deposition into responses essential for maintenance of homeostasis such as plasma and blood protein synthesis, mucous production, repair of the gastrointestinal tract mucosal integrity and maintenance of host defences (MacRae, 1993). The magnitude of these responses has not been fully assessed but calculations have suggested that parasitised sheep may need to synthesise an additional 50 grams of protein per day (Coop and Kyriazakis, 1999).

The absence of an effect of parasitism on lamb birth weight (Leyva *et al.*, 1982, Thomas and Ali, 1983) is in agreement with the higher priority of the reproductive effort over the expression of immunity to GINs. The proposed nutritional basis for the PPR is thus that it occurs due to an increased nutrient requirement of the prioritised reproductive effort at times when nutrient supply is scarce (Coop and Kyriazakis, 1999).

The periparturient ewe has a relatively high requirement for metabolisable protein (MP), and MP can be considered a scarce nutrient since voluntary intake is usually insufficient to meet MP requirement during late pregnancy and lactation (AFRC, 1993). The expression

of immunity also requires MP. It has been shown that an increased supply of MP enhances the expression of immunity of parasitised growing lambs (Bown *et al.*, 1991a, van Houtert *et al.*, 1995). Many components of the immune system, such as mast cells and leukocytes, are proteinaceous in nature and are expected to draw on MP resources (MacRae, 1993; Coop and Holmes, 1996). In addition, MP needs to be directed towards protein synthesis to counterbalance protein losses, which generally occur during parasitic infections (Coop and Holmes, 1996).

van Houtert *et al.* (1995) found that supplementation of a hay diet with 'protected' protein (50 or 100g fishmeal per day) affected the rate of worm expulsion after 10 weeks trickle infection with *T. columbriformis* and that the apparent rate of worm expulsion was related to the level of supplementation. Wallace *et al.* (1995) found no difference in the number of worms carried by sheep infected with *H. contortus*, but supplementation of a basal diet with soyabean meal had reduced worm fecundity. The responses to supplementation have been particularly effective where the protein was 'protected' (i.e. rumen undegradable), such as fishmeal (Coop and Kyriazakis, 1999; Donaldson *et al.,* 1998; 2001).

van Houtert *et al.* (1995) found that fishmeal supplementation resulted in an increase in the concentration of mast cell proteinases, and elevation in the levels of circulating eosinophils; however, these were not accompanied by changes in the levels of circulating specific and non-specific antibodies. Bown *et al.* (1991) attempted to separate the effects of protein and energy supply on the resilience of growing lambs to a trickle infection of *T. columbriformis*. They found that the protein but not the energy supplementation maintained the resilience of lambs to infection.

There are a few contradictory studies where no improvement in resilience followed increases in protein intake described in Coop *et al.* (1995). However, protein may not have been the first limiting (scarce) resource, and therefore, the absence of improvement in resilience is perhaps not surprising (Coop and Kyriazakis, 1999).

Coop & Kyriazakis (1999) suggested that the effect of protein supplementation may be related to the availability of certain amino-acids in their study supplementing lambs with 'protected' methionine. Most research has concentrated on the effects of host protein nutrition on their resilience and resistance to GIN infection. In most instances protein has been dealt with in its broad sense, although it is most likely that the requirements of the immune system are higher for some specific amino-acids than others (MacRae, 1993). Investigations considering both protein quantity and quality are needed when addressing its interactions with parasitism (MacRae, 1993).

Donaldson *et al.* (1998; 2001) found that supplementing the periparturient ewe's diet with fish-meal could reduce faecal worm egg output, but they were unable to state indisputably that this was due to protein supplementation as opposed to some other component in fish meal, for example, lipids.

Nutritional supplementation is of great interest, but continuous supplementation is possibly difficult to justify economically for commercial sheep production. It is of practical importance to sheep producers to know if expensive supplementation has longer term benefits for worm control and sheep productivity (Gray, 1997), especially in the UK with the sheep market as it is at the moment. Indeed, reducing the need for nematode control through improved nutrition will increase production costs as the level of nutrition required is above the normal levels required by the production system. However, if this was not so then there would be no nematode problem in the first place (Barger, 1997). Somehow, there has to be hope that the expense of the extra nutrition can be replaced by the income from increased production. The relevant comparison for the cost of novel control methods is not only that of anthelmintic-based control, but the risks and costs of uncontrolled parasitism.

1.6. Machine milking and the dairy ewe

The majority of sheep systems in the world produce meat and wool. However, in much of Asia and Europe, ewe's milk is an important source of dietary protein in the human diet and is also used to make high-quality cheeses (Treacher and Caja, 2002). The machine-milked ewe is pushed to her upper biological limits, according to Cant *et al.* (2001), whether this is mammary capacity, nutritional/digestive capacity or adipose responsiveness. The dairy ewe in early-lactation is generally unable to consume enough feed to meet her energy requirements (Cant *et al.*, 2001). Although, Cant *et al.* (2001) also stated that ewes suckling twin lambs produce more milk than the totally machine-milked ewe in the first 30 days. Machine milking enables an accurate measurement of milk produced as accurate measurements of milk production/lamb intake are difficult when suckling lambs (Treacher and Caja, 2001).

1.7. Conclusion

With the current concerns about farming practises, in particular the use of chemicals in the food chain and environment, and the ever-increasing anthelmintic resistance in nematodes, there is a need for a more sustainable and chemical-free method of reducing gastro-intestinal nematode infections in ruminants. Therefore, as the periparturient ewe is a major source of nematodes in lambs (Heath and Michel, 1969; Jackson *et al.*, 1988), it is important to reduce her faecal nematode egg output, which often increases around parturition. Nutrition of the periparturient ewe appears to be a possible way of reducing her faecal egg output and creating a more sustainable nematode control strategy. Although Donaldson *et al.* (1998; 2001) had positive results with fishmeal supplementation, fishmeal cannot be used in the UK now, due to the emergence of BSE and the subsequent worries over feeding animal and fish-meals to herbivores. Therefore, another source of protein is necessary or it may even be necessary to deduce what exactly, in the fishmeal, had an effect on the FECs.

CHAPTER TWO- ROUTINE ANALYSIS

2.1. *Feed analysis*

Samples of feeds were collected and stored at –20°C at weekly intervals for the subsequent analysis of fatty acid content (Experiment One only), and proximate analysis of dry matter (DM), crude protein (CP), organic matter (OM), ether extract as described by AOAC (1990), and neutral detergent fibre (NDF) as described by Van Soest *et al.* (1991).

2.1.1. Dry matter

Sub-samples of all concentrate rations and hay were analysed for dry matter content (g kg^{-1} fresh weight) by drying in an oven at 100°C to a constant weight.

2.1.2. Organic matter

Between 1 and 2 grams of dried samples of feed were accurately weighed and placed in porcelain crucibles and then heated at 550°C for 4 hours in a muffle furnace (Fissons). The samples were then cooled in a dessicator and reweighed. The organic matter of the sample was calculated as 1000-ash residue (g kg^{-1} DM) (AOAC, 1990).

2.1.3. Ether extract

Two to three grams of ground fresh feed sample were weighed into pre-weighed cellulose extraction thimbles, and plugged with defatted cotton wool. The total fat was extracted by boiling the samples in 25ml of petroleum ether for 30 minutes. The thimbles were then rinsed for 15 minutes and the petroleum ether allowed to evaporate. The fat content was then calculated as:

$$\text{Ether extract (g kg}^{-1}\text{ DM)} = \frac{\text{weight of fat (g)}}{\text{weight of sample}} \times 1000$$

2.1.4. Neutral detergent fibre (NDF)

A 0.5 g sub-sample of dried feed sample was placed into a previously weighed crucible. This was then placed into the Fibertec apparatus (Tecator, Foss UK 1020) and 25 ml of cold neutral detergent reagent (a solution of 93g of disodium ethylene diamine tetra-acetate dihydrate (EDTA) and 34g sodium borate [$Na_2B_4O_7.10H_2O$] in distilled water dissolved by gentle heating to which 150g sodium lauryl sulphate and 50ml of 2-ethoxy ethanol was added and a second solution made up by dissolving 22.8g of anhydrous disodium hydrogen phosphate [NA_2HPO_4] in distilled water. Both solutions were then mixed and diluted to 5 litres with the pH adjusted to approximately 6.9-7.1), and 0.5ml octanol (an anti-foaming agent) were added and brought to the boil for 30 minutes on the fibertec condenser. The heat was turned off and 25ml of cold neutral detergent reagent plus 2ml of α-amylase solution (2.0g of α-amylase [BDH] in 90ml distilled water, filtered and then to filtrate added 10ml of 2-ethoxyl ethanol) were added a boiled for a further 30 minutes. The digest was then filtered and washed three times with 20ml of hot distilled water. At the end of filtration 25ml of hot distilled water (80°C) and 2 ml of α-amylase was added, the sample mixed and left to stand for 15 minutes. The sample was then filtered and washed a further three times with hot distilled water and once with acetone. The crucible and sample were then dried at 100°C overnight and weighed then ashed at 550°C for 4 hours, cooled in a dessicator and reweighed.

The NDF content (g kg^{-1} DM) was calculated as:

$$\text{NDF g kg}^{-1}\text{ DM} = \frac{\text{residue weight (g)} - \text{ash weight (g)}}{\text{Sample weight (gDM)}} \times 1000$$

2.1.5 Crude protein

Nitrogen analysis was performed on a LECO FP528 (LECO Corp. St. Joseph, M.I. USA) using the Dumas method (Watson and Galliher, 2001). Approximately 0.15g of feed

sample was accurately weighed, wrapped in a small foil ball. The foil balls were heated to 1020°C in a mixture of O_2 and CO_2 and the resulting nitrous oxides were reduced to N_2 by warmed copper filings and the N measured with a thermal conductivity detector. To convert to crude protein a factor of 6.25 x N was used.

2.2. *Fatty acid analysis of the treatment diets (Experiment 1)*

Three hundred milligrams of ground feed samples were accurately weighed into 30ml soveril tubes in duplicate. Six millilitres of 5M saponification mixture (140.3g KOH dissolved in 250mls of distilled water, mixed with 250mls of Methanol) and 200µl of C21 standard (200µl @ concentration 7.548mg ml^{-1} therefore = 1.5096) were added. Saponification was performed for 3hours at 60°C shaking regularly. The samples were then cooled and 3ml 10N H_2SO_4 added gently swirling and releasing pressure in the meanwhile. They were then hydrolysed for a further hour at 60°C and cooled.

Twelve millilitres of water and 5ml petrol spirit were added. Then the tubes were vigorously shaken for 1 minute and centrifuged at 2000rpm for 5 minutes. The top layer was transferred to a clean tube. This was repeated twice, leaving three top layers in the tube. This was then reduced under nitrogen to approximately 10ml. Sodium hydrogen carbonate was added to the reduced solution until effervescing stopped and a large spatula of anhydrous sodium sulphate and shaken. This was then centrifuged at 2000rpm for a further 5 minutes. The residue (pH checked to be 1) was then poured into a clean tube, dried under nitrogen and stored at -18°C until methylation.

2.2.1. Methylation of samples

Samples were warmed at room temperature, and then the tube sides washed with less than 0.5ml of 40:60 petrol ether (PE). In a fume cupboard with an open bottle of glacial acetic acid in it added approximately 12 drops of diazamethane to each of the tubes or until the samples appeared yellow. The fume cupboard was then closed for 10 minutes then the rack

of tubes was placed into a water bath for 5 minutes until all the diazamethane was evaporated. The tubes were removed individually from the waterbath, dried briefly under nitrogen then 1.5ml PE was added and capped and stored at -18°C until being run on the gas-solid chromatographer (GC).

2.3. Treatment of foot rot

Foot rot was treated *post-partum* to prevent stressing the ewes during late pregnancy. The ewes were foot trimmed and sprayed with oxytetracycline *post partum* and passed through a footbath which contained zinc sulphate twice a day for approximately two to five minutes.

2.3.1 Live weights and condition scores

Ewe live weights (LW) and condition scores (CS) were determined weekly at the same time (Wednesday 2pm). Body CS was estimated by palpation of the lumbar vertebrae, transverse and longitudinal processes as described by Russel (1984) by the same person over the duration of all experiments. Ewe LW were performed using an electronic EziWeigh 1 livestock scales (Tru-Test Ltd, Auckland, New Zealand) using standard weights to calibrate the machine each weighing.

2.3.2 Lamb birth weights and husbandry

Lamb birth weights were taken using electronic scales when dry within 12 hours of birth. The lambs were removed from the ewe and reared on milk replacer after 72 hours for experiments 1 and 2 and the lambs from the milking treatments for experiment 3. For the suckling treatments, litters with more than two lambs had the remainder removed and those lambs were either fostered immediately at birth to single-lamb bearing ewes or removed at 72 hours, in order that each ewe reared two lambs where possible regardless of litter carried through pregnancy.

2.4. *Parasitology*

2.4.1. Modified McMaster floatation technique for faecal egg count analysis (Experiment 1)

Four grams of faeces were placed in a mortar to which 30ml of water was added. The mixture was ground using a pestle. Enough suspension was removed to fill two capped 1.5ml micro centrifuge tubes. These were then mixed and placed in a centrifuge at 4000rpm for 4 minutes after which the supernatant was discarded. The tubes were then filled with saturated sodium chloride solution and mixed thoroughly. Using a bulb pipette the solution was used to fill both chambers of a McMaster counting slide and examined under the microscope. The eggs from both counts were then multiplied by 22.67 to give the number of eggs per gram (EPG) of faeces.

2.4.2. Moredun modified floatation technique for faecal egg counting

A rectal faecal sample of between one and ten grams was obtained and placed in a 100 ml sample pot (Starstedt Ltd, Leicester, UK). Between three and five grams were placed in a 150 x 100 mm polygrip bag, 10ml of water added per gram of faeces and then emulsified using a stomacher (Seward Medical, London, UK). A 10 ml sub-sample was pipetted and passed over a 1mm sieve and washed through with five millilitres of tap water, and the retentate pressed to extract most of the fluid. The filtrate was then poured into a polyallomer centrifuge tube (Beckmann Coulter Ltd, High Wycombe, Bucks, UK) and centrifuged at 1000 rpm (100g) for two minutes.

The supernate was removed using a vacuum line, leaving 1ml of faecal debris and nematode eggs at the bottom; this was then resuspended in 10ml of saturated sodium chloride solution (heated H_2O with NaCl added until saturation), mixed and centrifuged at 100g for two minutes. Artery forceps (Carmault forces [straight] Cox Surgical, Surrey, UK) were used to clamp the tube off just below the meniscus and the contents of the upper chamber (two – three millilitres) were poured into a four millilitre polystyrene macro

disposable cuvette (Fisher Scientific Ltd, Loughborough, UK). The upper chamber was then rinsed with 1ml of saturated sodium chloride and added to the cuvette. The cuvette was then inverted to resuspend the eggs uniformly and filled and sealed with a cuvette cap (Elkay Laboratories, Basingstoke, UK). Each cuvette was assumed to contain all the eggs recovered from one gram of faeces. Counting was performed under the lowest magnification (x40) using a compound microscope with a mechanical stage and Kellner eyepiece fitted with a 21mm Miller Square Graticule (Graticules Ltd., Kent, UK). The cuvette was placed on a microscope slide and by using the mechanical stage two longitudinal traverses along the cuvette were performed counting the number of eggs which fell within either the large or small square of the eye piece graticule. If less than three eggs were counted in each traverse then the whole cuvette would be counted. The total was multiplied by three if the large square was used and by nine if the small square was used, to obtain an estimate of the number of eggs per gram.

2.4.3. Removal of the abomasum for worm recovery

Slaughter was performed by electric stunning and exsanguination at a commercial abattoir (Malpas abattoir, Cheshire – Experiment Two and Handley abattoir, Staffordshire – Experiment Three). The entire abomasum was removed and with the contents were placed in a labelled 10 litre bucket (The Home Brew Shop, Farnborough, UK). The abomasum was then cut and a scraping of the mucosa taken and placed on solid CO_2 and a slice of mucosal membrane taken and placed in 4% paraformaldehyde solution (four grams paraformaldehyde powder dissolved in 100ml phosphate buffered saline (PBS)). The abomasum was then washed using up to five litres of warm, 0.85% saline solution. This mix of abomasum contents and washings was then topped up with saline solution to five litres and a 500ml sub-sample removed and preserved with formaldehyde to form a 2% formaldehyde solution.

The washed abomasum was then placed in 1.5 litres of 1% w/v solution of pepsin/HCl to provide a pepsin digest and incubated at 37°C for 4-6 hours. The samples were agitated at every hour to ensure complete digestion. The mucosa was then sloughed off using gloved hands and the fluid retained was sub-sampled and preserved in a 2% formaldehyde solution.

2.4.4. Preparation of the abomasal samples for worm burden estimation

The 10% aliquot of either the abomasal washings or the digests were sub-divided into 4 sub-samples, to which 1.5ml of helminthological iodine (potassium iodide (KI_2)) was added to 1 of the sub-samples (2.5% of total abomasal sample) and allowed to stain for a few minutes before then being passed over a 38μm stainless steel sieve to remove most of the iodine and any fine debris. The retentate was then washed off the sieve and mixed thoroughly before a small quantity was dispensed and placed on a contact plate (Bibby-Sterilin, UK). The sample was then examined and the worms counted and collected with a mounted needle and placed in 2% formalin in bijoux labelled male, female or immature. The total number of worms was estimated by multiplying the total recovered by 100/sample size (e.g. if 5% total abomasal sample had been counted then it would be n x (100/5) or n x 20.

2.4.5 Determination of mucosal mast cells and eosinophil numbers from the abomasal mucosa.

Mucosal mast cell and eosinophil counts were done on sections of an abomasal fold using the techniques described by Huntley *et al.* (1992). The abomasal fold samples were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4). After fixation of 6 hours, the samples were transferred into 90% ethanol and stored at 4°C until processing. The samples were dehydrated, embedded, sectioned and stained using Toluidine blue and Carbol chromatrope by Moredun Research Institute, Scotland using methods described by Huntley *et al.*

(1992). The stained cells were enumerated using a x10 eye piece containing a calibrated (using a slide micrometre – Graticules Ltd, Kent.) graticule and a x 40 objective lens encompassing an area of 265 x 265µm. Counts were made on a minimum of 10 graticule fields from 3 separate sections of the folds and were expressed as cells per $0.2mm^2$.

2.4.6. *In Utero* nematode egg counts

Twenty female worms per ewe were randomly selected and placed on glass microscope slides. A drop of lacto-phenol (20ml water, 40ml glycerine, 20ml Lactic acid and 20ml Phenol, melted crystals) was added and a cover slip applied. The worms were examined for eggs *in utero* after 24 hours using a microscope with a x 40 objective.

2.5. *Blood analysis*

Blood samples collected by jugular venepuncture into 7ml evacuated tubes (containing the anticoagulants – lithium heparin, Ethylenediaminetetracetate (EDTA) and potassium oxalate) (Vacutainers systems – Becton Dickinson France) were centrifuged at 3000rpm (1610 x g) for 15 minutes (Beckman Avanti 30 centrifuge) and the plasma stored in capped 1.5ml micro-centrifuge tubes at -20°C until use. Tubes that contained no anticoagulant were stored until the next day before being centrifuged at 3000rpm (1610 x g) for 15 minutes and the serum removed and stored in capped 1.5ml micro-centrifuge tubes at -20°C.

2.5.1. Peripheral eosinophil counts

Peripheral eosinophil cell counts were compared on a fortnightly basis. A 50µl sample of whole blood (Heparinised samples in Experiments One and Two and EDTA samples in Experiment 3) was added to 450µl Carpentiers eosinophil counting solution (0.4ml of 2% aqueous Eosin Y and 0.6ml $CaCO_3$ saturated 40% formaldehyde and made up to 20ml with distilled H_2O which was made up immediately prior to use). Cells were stained at room

temperature and then counted on a Fast-read Chamber (Immune systems Ltd, Paignton, Devon, UK), under a microscope at 40x magnification. The number of eosinophils that fell within the grid pattern were counted and the total number divided by 100 to give the number of eosinophils $x10^9$/L.

2.5.2. Blood metabolite analysis

Plasma samples from the lithium heparinised tubes were analysed for urea, total protein and albumin whilst samples from potassium oxalate tubes were analysed for glucose and β-hydroxybutyrate. Analysis was performed on a Bayer Technicon RA-1000 auto-analyser (Bayer plc, Newbury, Berks, UK) using the methods of Sigma (Sigma Diagnostics, St Louis, USA)

2.5.3. Haematology

Whole blood samples from EDTA evacuated tubes were analysed within 2 hours of collection using a Vet ABC™ animal blood counter (Scil Animal Care Company, Viernheim c/o ABX Diagnostics, Montpelier, France). The samples were gently agitated for 3 minutes immediately prior to analysis. On the day of analysis, the blood counter was calibrated using manufacturer standards, Minotrol 16, (ABX Diagnostics, Montpelier, France). The ewe red blood cells (RBC), white blood cells (WBC) number, haemoglobin (Hb) concentration and packed cell volumes (PCV; haematocrit) (Experiments Two and Three) were recorded.

2.5.4. Packed Cell Volumes (Experiment 1)

Whole blood from heparinised evacuated tubes (Becton, Dickinson and Company, Vacutainer Systems, Plymouth, UK) was introduced into a heparinised capillary tube (Hawksley & Sons Ltd, Lancing, Sussex, UK) by capillary action and bunged with

plasticine. Samples were then centrifuged in a haematocrit centrifuge at 7000rpm for 15 minutes and the percentage PCV measured using a haematocrit reader.

2.5.5. Determination of antibodies to *Teladorsagia circumcincta* infective stage larvae (Experiment One)

Enzyme Linked Immunosorbent Assays (ELISAs) were performed on the ewe sera at Moredun Research Institute, Penicuik, Scotland. The samples were analysed for immunoglobulin A (IgA), immunoglobulin E (IgE) and immunoglobulin G (IgG) antibodies against *Teladorsagia circumcincta* infective 3rd stage larvae as described by Huntley *et al.* (1998).

2.6. *Milk analysis*

Milk samples were stored at -20°C after collection then thawed in a water bath at 40°C immediately prior to analysis on a Dairy Lab 2 double-beam infra-red spectrophotometer (Multispec Ltd, York, UK.) to determine fat, crude protein, lactose and solids-non-fat (SNF; g kg^{-1}). The Dairy Lab 2 was calibrated using standards containing known concentrations of fat (Quality Management, Trenslo House, Bury, UK). The homogenizer and sampling probe were washed with warm (40°C) deionised water between samples.

CHAPTER THREE – EXPERIMENT ONE

THE EFFECTS OF METABOLISABLE PROTEIN AND FISH OIL SUPPLY ON THE PERIPARTURIENT RISE IN FAECAL EGG COUNTS IN DAIRY EWES

3.1 *Introduction*

Previous studies have shown that the provision of fish-meal during the periparturient period can reduce faecal egg output (Donaldson *et al.*, 1998; 2001). Because the ruminally protected protein source used in the studies of Donaldson *et al.* (2001) was derived from fish-meal, the effects may have been due to the post-ruminal supplementation of polyunsaturated fish oils, for example eicosapentaeoic acid (EPA) and docosahexaenoic acid (DHA), as well as the additional protein. Manipulation of the fatty acid content of membrane lipids may offer an opportunity to modulate the nature of the immune response (Muturi, 2003). Also due to the unsuitability of fish-meal as an animal food source since the Bovine Spongiform Encephalopathy (BSE) outbreak in 1987, it was necessary to find other suitable protein sources.

Due to previous evidence that ewes rearing twin lambs rather than single lambs suffered a more pronounced PPR in FECs (Donaldson *et al.*, 1998), it was of merit to study the effects of the MP source and fish oil on the PPR in machine milked, dairy ewes. This would provide a higher milk production rate in the first two weeks of lactation than ewes suckling twins. According to Cant *et al.* (2001), machine milking can cause the ewe to exceed her 'upper biological limits' and as a consequence, in early lactation is unable to consume enough food to meet energy demands. Machine milking would also facilitate the accurate measurement of milk yield which could assist in quantifying nutrient requirements for milk production and its inter-relationship with nematodes and the PPR.

The objectives of this study were to examine the effects of increased protein and fish oil supply on the performance and faecal nematode egg output of periparturient dairy ewes infected with *Teladorsagia circumcincta.*

3.2 *Material and methods*

3.2.1 Animals and experimental design

Fifty Friesland ewes were implanted with progestagen-impregnated intra-vaginal sponges (Chronogest; Intervet Ltd, Holland) to synchronise oestrus and result in an estimated mean parturition date of 12th March 2001. The ewes were ultra-sound scanned at 84 days of gestation to confirm pregnancy status. At 17 weeks prior to parturition, the ewes were housed and dosed with Panacur™ (Fenbendazole); (Hoechst Roussel Vet Ltd., Milton Keynes, UK), at the manufacturer's recommended dose (5mg kg^{-1} live weight). The ewes were offered wheat straw *ad-libitum* and remained group-housed until the start of the experiment at five weeks prior to parturition. The ewes were injected with Heptavac-P plus™ vaccine, (Hoechst Roussel Vet Ltd., Milton Keynes, UK), a combined 7 in 1 Clostridial plus Pasterella vaccine for the active immunisation of sheep, 4 weeks prior to parturition.

Thirty-two ewes were selected for use in the experiment (20 twin-bearing ewes, 8 triplet-bearing ewes and 4 single-lamb bearing) of varying ages ranging from 2 to 9 years. The ewes were blocked according to foetal number, live weight (LW), condition score (CS), age and previous lactation yields and allocated to one of 4 treatment diets (n=8, consisting of 1 single-, 2 triplet- and 5 twin-bearing ewes). The treatment groups were - Low MP not including Fish oil (LMP-), Low MP including Fish oil (LMP+), High MP not including Fish oil (HMP-), High MP including Fish oil (HMP+). The diets were fed as a complete diet based on chopped hay, barley, and molassed sugar beet pulp (Tables 3.1 and 3.2). The ewes were individually housed in 3m^2 pens, bedded on shavings and water was made available *ad-libitum*.

The ewes were offered the dietary treatments from 5 weeks prior to parturition until week 8 of lactation. *Pre-partum* feeding levels were restricted according to ME requirements for litter size, and an estimated live weight gain of 0.05kg day^{-1} according to AFRC (1993). *Post-partum*, all ewes were offered a restricted level of feed on the assumption that they

were producing 2.5 litres of milk and losing 0.05kg live weight day^{-1} (AFRC, 1993). Equal amounts of the ration were offered at 08.00 hours and 16.00 hours. Lamb removal and the recording of birth weight are described in section 2.3.2.

Table 3.1 *Diet composition of the diets that were fed to the Friesland dairy ewes during the* pre-partum *period.*

(g kg^{-1} fresh weight)	LMP – fish oil	LMP + fish oil	HMP – fish oil	HMP + fish oil
Ground hay	345	345	345	345
Ground barley	383	370	315	305
Molassed sugar beet pulp	203	198	165	158
Lactamine™*	0	0	104	104
Soya bean meal (SBM)	0	0	0	0
Rape seed meal (RSM)	0	0	0	0
Fish oil*	0	36	0	36
Megalac™**	18	0	18	0
Urea	13	13	13	13
Mins/vits	17	17	17	17
Molasses	23	23	23	23
Total	1000	1000	1000	1000
Predicted diet composition				
ME MJ kg^{-1} DM	11.6	11.4	11.5	11.3
CP g kg^{-1} DM	141	139	183	181
ERDP 5[1]	103	101	104	102
DUP 5[1]	17	17	53	53
ERDP/FME ratio	10	10	10	10
MP g kg^{-1}DM	83	82	120	119

[1] calculated at a rumen solid phase outflow rate of 0.05 h^{-1} (g kg^{-1}) (AFRC, 1993)

* Lactamine™ (a source of protected metabolisable protein in the form of protected SBM (sopralin) and protected amino acids including methionine mixed with RSM and SBM) and fish oil mixed with vermiculite, both provided by Trouw nutrition, Northwich, UK.

** Megalac™ a protected fat source (Volac, Royston, UK)

ME = Metabolisable energy, CP = Crude protein, ERDP = Effective rumen degradable protein, DUP = Digestible undegradable protein, FME = Fermentable metabolisable energy, MP = Metabolisable protein.

Table 3.2 *Diet composition of the diets that were fed to the Friesland dairy ewes during the* post-artum *period.*

($g\ kg^{-1}$ fresh weight)	LMP – fish oil	LMP + fish oil	HMP – fish oil	HMP + fish oil
Hay	350	355	352.5	357.5
Ground Barley	320	307	225	210
Molassed Sugar beet pulp	173	165	120	115
Lactamine ™*	0	0	148	148
Soya bean meal	37	37	37	37
Rape seed meal	50	50	50	50
Fish oil mix*	0	35	0	35
Megalac™**	18	0	18	0
Urea	11	11	8.3	8.3
Mins/vits	17	17	17	17
Molasses	23	23	23	23
Total	1000	1000	1000	1000
Predicted diet composition				
ME MJ kg^{-1} DM	11.7	11.5	11.6	11.4
CP g kg^{-1} DM	177	174	228	225
ERDP 8[1]	238	239	234	234
DUP 8[1]	73	74	175	170
ERDP/FME ratio	11	11	11	11
MP g kg^{-1} DM	225	225	323	323

[1] calculated at a rumen solid phase outflow rate of 0.08 h^{-1} ($g\ kg^{-1}$) (AFRC, 1993)

* Lactamine™ (a source of protected metabolisable protein in the form of protected SBM (sopralin) and protected amino acids including methionine mixed with RSM and SBM) and fish oil mixed with vermiculite, both provided by Trouw nutrition, Northwich, UK.

** Megalac™ a protected fat source (Volac, Royston, UK)

3.2.2. Parasite challenge

Parasite inoculation involved administration of approximately 14,000 *Teladorsagia circumcincta* (Moredun Ovine Anthelmintic Susceptible Isolates; Moredun Research Institute, Scotland, UK.), infective larvae (L_3) per week from week 1 *post-partum*. This was achieved by placing 5ml of the L_3 and water suspension (approximately 4,600 larvae per 5ml), on a Whatman™ 1 filter paper of 110mm diameter (Whatman International Ltd., Maidstone, UK). The paper was then rolled into a small pellet and administered orally using a bolus applicator device (Ivomec Maximizer™) as soon as possible to prevent drying out. This was performed three times a week at approximately two hours after the morning feed. The L_3 suspension was stored at approximately 4°C in water and a new

batch provided after 4 weeks. The larvae were examined under a microscope at 10 x magnification to monitor motility and viability.

3.2.3. Sample collection

Faecal samples of between 1 and 10 grams were collected from the rectum at 2pm every Friday from one week prior to parturition and placed in 100ml sample pots (Starstedt Ltd, Leicester, UK) and refrigerated at 4°C. Faecal egg counts were performed within 3 days of sample collection. Faecal egg counts (FECs) were estimated using the modified McMaster procedure (Section 2.4.1; Christie and Jackson, 1982).

Blood samples were collected by jugular venepuncture into 7ml evacuated tubes containing either heparin, potassium oxalate or additive free (Becton, Dickinson and Company, Vacutainer Systems, Plymouth, UK), approximately 2 hours after the morning feed, fortnightly *pre-partum* and weekly *post-partum* and analysed for peripheral eosinophil number, blood metabolite concentrations, packed cell volume (PCV) and anti-*T. circumcincta* L_3 immunoglobulin levels (Sections 2.5 to 2.5.5 excluding 2.5.3).

The ewes were milked twice daily in a Fullwood automatic goat-milking parlour. Milk yield was recorded from two consecutive evening (16.00h) and morning milking (07.30h) each week and samples collected and placed in 20ml capped milk tubes (Massmould, Luton, Bedfordshire, UK), and stored at -20°C until analysis (Section 2.6.). Ewe live weights and condition scores were recorded on the same day each week at 2pm. Samples of the treatment diets were collected weekly and stored in air tight bags at -20°C until analysis (described in sections 2.1 & 2.2), the results are presented in Tables 3.1 to 3.6.

3.2.4. Statistical analysis

Skewed data was transformed by Log_{10} (n+1). Statistical analysis was performed on Genstat release 5.1. Lawes Agricultural Trust (1995) and analysed as a 2 x 2 factorial

analysis of variance (ANOVA), using the factors protein level (MP), fish oil inclusion (fish) and their interaction (int).

3.3 *Results*

3.3.1. Feed analysis results

The proximal analysis of the *pre-* and *post-partum* diets confirmed that the ewes offered the HMP treatments were fed higher crude protein levels than the LMP treated ewes. The analysis suggested that the HMP + fish oil treatment had less ether extract than the HMP – fish oil treatment both *pre-* and *post-partum* (Tables 3.3. and 3.4.). The fatty acid analysis demonstrated a clear difference in EPA between the treatments including fish oil and the non fish oil treatments however there was not such a clear distinction in DHA concentrations between the treatments (Tables 3.5. and 3.6.).

Table 3.3. *Proximate analysis of the diets that were fed to the Friesland dairy ewes during the* pre-partum *period.*

	LMP - fish oil	LMP + fish oil	HMP - fish oil	HMP + fish oil
Dry matter (g kg^{-1} fresh)	853	847	844	844
Protein (g kg^{-1} DM)	153	139	210	196
Ash (g kg^{-1} DM)	78	101	101	94
Ether extract (g kg^{-1} DM)	21	24	32	24
Neutral detergent fibre (g kg^{-1} DM)	381	418	393	387

Table 3.4. *Proximate analysis of the diets that were fed to the Friesland dairy ewes during the* post-partum *period.*

	LMP - fish oil	LMP + fish oil	HMP – fish oil	HMP + fish oil
Dry matter (g kg^{-1} fresh)	850	852	858	859
Protein (g kg^{-1} DM)	173	181	210	233
Ash (g kg^{-1} DM)	75	98	84	99
Ether extract (g kg^{-1} DM)	24	28	29	24
Neutral detergent fibre (g kg^{-1}DM)	392	378	422	379

Table 3.5. *The fatty acid profile of the diets fed to the Friesland dairy ewes during the* pre-partum *period (g kg^{-1} DM).*

Fatty acid (FA)	LMP - fish oil	LMP + fish oil	HMP - fish oil	HMP +fish oil
C12:0	0.50	0.40	0.71	0.37
C14:0	0.55	1.32	0.72	1.50
C16:0	12.9	6.51	14.8	8.19
C16:1	0.17	1.06	0.31	1.18
C18:0	1.14	1.14	2.60	2.34
C18:1 n-9	7.59	4.29	8.65	4.99
C18:1 n-7	0.32	0.75	0.66	0.82
C18:2 n-6	11.8	6.16	8.56	6.83
C18:3 n-3	1.36	0.90	1.14	1.09
C20:1	0.18	1.10	0.20	1.26
C20:5 n-3 (EPA)	0.10	1.10	0.16	1.30
C22:6 n-3 (DHA)	0.10	0.33	0.32	0.50
Remaining fatty acids	2.75	9.87	4.57	6.65
Total FAs in sample	38.8	34.9	41.9	37.0

Table 3.6 *The fatty acid profile of the diets fed to the Friesland dairy ewes during the* post-partum *period (g kg^{-1} DM).*

Fatty acid	LMP – fish oil	LMP + fish oil	HMP – fish oil	HMP + fish oil
C12:0	0.77	0.63	0.56	0.46
C14:0	0.62	1.90	0.58	1.39
C16:0	13.6	7.80	11.39	7.86
C16:1	0.29	1.74	0.36	1.15
C18:0	1.43	1.43	1.79	1.89
C18:1 n-9	9.18	6.98	7.29	5.91
C18:1 n-7	0.84	1.33	0.78	1.04
C18:2 n-6	8.86	7.84	7.86	7.29
C18:3 n-3	1.34	1.37	1.16	1.20
C20:1	0.09	1.69	0.40	1.23
C20:5 n-3 (EPA)	0.26	1.85	0.41	1.29
C22:6 n-3 (DHA)	0.25	0.51	0.19	0.27
Remaining fatty acids	4.02	8.64	8.28	6.66
Total FAs in sample	40.1	43.7	39.3	37.6

3.3.2. Sheep health

There was no indication of clinical teladorsagiasis throughout the experimental period, as demonstrated by the ewes appearing healthy and producing normal faeces along with the low faecal egg counts and later confirmed by normal plasma albumin levels as hypoalbuminaemia is a sign of protein leakage into the abomasum due to parasitic damage

to the mucosa (Kassai, 1999). Twenty-seven of the 32 ewes had foot rot from the onset of the experiment but all were treated by foot trimming, *post-partum*. A zinc sulphate footbath was also set up outside the parlour exit at the start of lactation; by the end of the experiment all the ewes were free of foot rot.

One ewe aborted sixteen days before the expected parturition date and was removed from the statistical analysis. One ewe in the HMP including fish oil group was omitted from the milk analysis due to very poor milk yields.

There were no interactions between MP and fish oil in any of the parameters measured and therefore only the main effects are presented.

3.3.2.1. *Ewe live weight and body condition scores*

Ewes offered the HMP treatments lost more LW ($P < 0.05$) during lactation than the ewes on the LMP treatments (Table 3.7). The dietary treatments had no effects on LW or CS during pregnancy or CS of the ewes during lactation.

Table 3.7 *The effect of increased MP supply and fish oil inclusion on ewe live weight (kg) and condition score.*

	LMP - fish oil	HMP - fish oil	LMP + fish oil	HMP + fish oil	s.e.d.	MP	Fish oil
Pre-partum LW (kg)							
Week -5	70.2	71.8	71.7	71.2	4.17	ns	ns
Week -1	74.9	79.5	78.2	77.3	5.34	ns	ns
Pre-partum change	+4.79	+7.71	+5.89	+6.14	2.02	ns	ns
Pre-partum CS							
Week -5	2.0	2.0	1.8	1.8	0.22	ns	ns
Week -1	1.6	1.8	1.6	1.7	0.16	ns	ns
Pre-partum change	-0.34	-0.16	-0.25	-0.22	0.15	ns	ns
Post-partum LW (kg)							
12 hr *post-partum*	61.8	65.2	62.9	63.7	3.89	ns	ns
Wk 8 *post-partum*	64.8	64.1	67.1	66.0	3.68	ns	ns
Post-partum change	+3.00	-1.03	+4.11	+2.33	1.66	*	ns
Post-partum CS							
12 hr *post-partum*	1.4	1.6	1.3	1.3	0.14	ns	ns
Wk 8 *post-partum*	1.5	1.6	1.5	1.6	0.14	ns	ns
Post-partum change	+0.09	0.00	+0.21	+0.22	0.13	ns	ns

*Key ns = non significant (P>0.05), *P<0.05, **P<0.01, *** P<0.001.*

3.3.2.2. *Gestation length and lamb birth weights*

Ewes offered diets supplemented with fish oil produced lambs with higher birth weights ($P<0.05$) than those ewes offered unsupplemented diets (Table 3.8). Increasing the MP supply had no effect on lamb birth weights. Ewes fed diets supplemented with fish oil had gestation lengths which were approximately 2 days longer ($P<0.05$) than the ewes not supplemented.

Table 3.8 *The effect of increased MP supply and fish oil inclusion on lamb birth weight and gestation length*

	LMP - fish oil	LMP + fish oil	HMP - fish oil	HMP + fish oil	S.E.D.	MP	fish
Gestation length (days)	144.8	146.7	146.0	147.5	0.90	ns	*
Lamb birth weight (kg)	3.65	4.24	3.92	4.12	0.25	ns	*

*Key ns = non significant (P>0.05), *P<0.05, **P<0.01, *** P<0.001.*

3.3.3. Parasitology results

3.3.3.1 *Faecal nematode egg counts*

The ewes were carrying a natural infection of 'strongyle type' nematodes (determined by the presence of thin-shelled, oval eggs of between 40 and 110μm long in the faeces) as faecal egg counts ranged between 272 and 465 epg prior to challenge with infective larvae in week one (Figure 3.1.). By week two *post-partum* (one week after challenge commenced) the worm egg counts had dropped to below 200 epg and remained below 500 epg for all four treatment groups for the rest of the challenge period. The transformed means showed no significant difference between the numbers of nematode eggs in the faeces of the ewes on any of the dietary treatments.

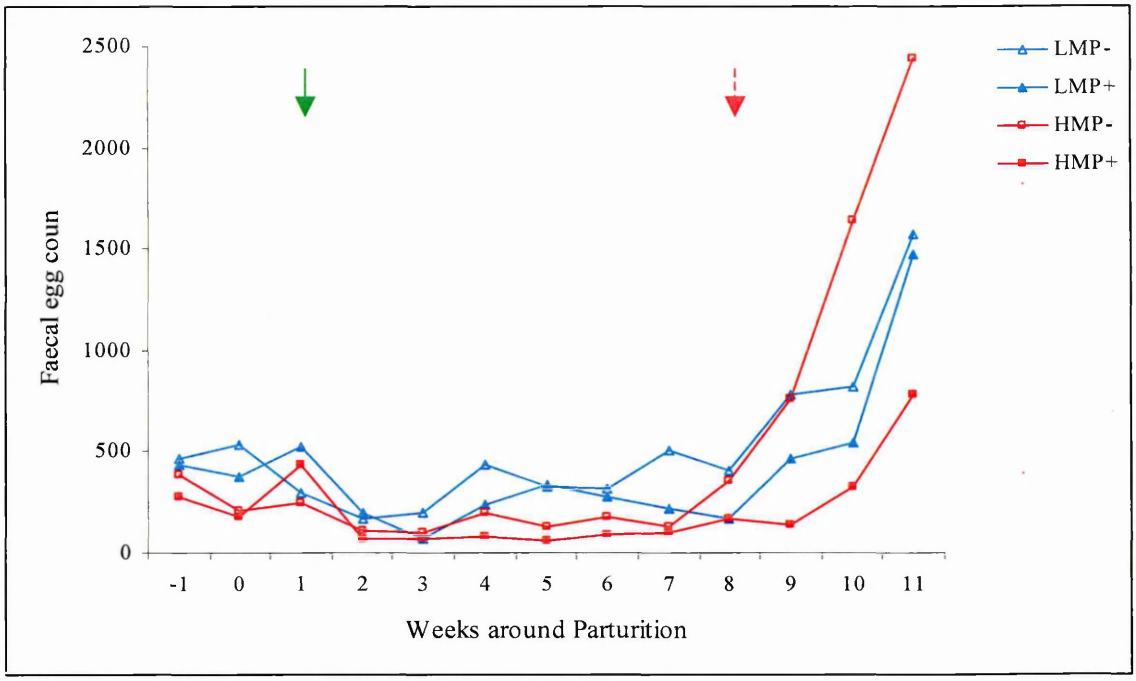


Figure 3.1. *The effect of increased MP supply and fish oil supplementation on the mean faecal nematode egg counts (the solid arrow indicates the start and the dashed arrow indicates finish of challenge with infective* T. circumcincta *larvae)*

3.3.4. Haematology

3.3.4.1 *Eosinophil counts*

Eosinophil counts were recorded immediately prior to inoculation with infective *T. circumcincta* larvae in week one (Figure 3.2.) and weekly thereafter. There was an initial rise in eosinophil number after challenge from an average across all treatments of 0.26 x 10^9/l whole blood pre-challenge to 2.28 x 10^9/l in week 3 of lactation with a steady decline thereafter. The fish oil supplemented ewes exhibited a greater number of eosinophils (an average of 2.76 x 10^9/litre) than the unsupplemented ewes (an average of 1.79 x 10^9/litre) in week 3 although the difference was not significant ($P > 0.05$). There were no significant differences in the ewe peripheral eosinophil counts between the treatment groups at any time point.

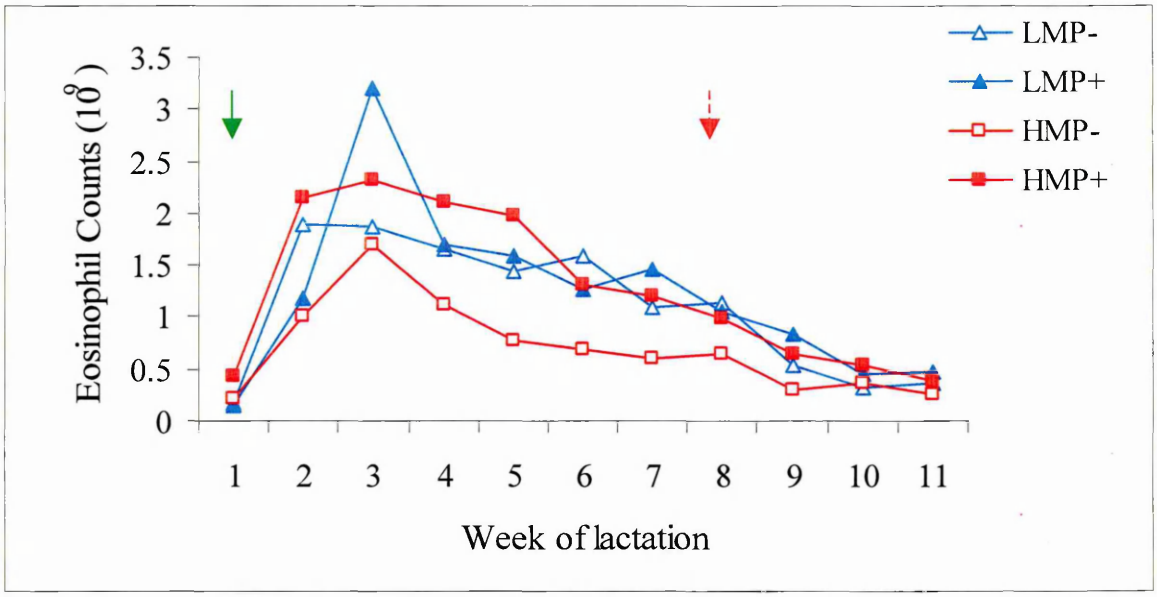


Figure 3.2 *The effect of increased MP supply and fish oil supplementation on the arithmetic mean ewe peripheral eosinophil count ($nx10^9$). (the solid arrow indicates the start and the dashed arrow indicates finish of challenge with infective* T. circumcincta *larvae).*

3.3.4.2 *Blood haematocrit concentration*

There were no significant differences in the ewe PCVs between the different treatment groups (Table 3.9).

Table 3.9. *The effect of increased MP supply and fish oil supplementation on the mean ewe haematocrit levels.*

	LMP - fish oil	LMP + fish oil	HMP - fish oil	HMP + fish oil	S.E.D.	MP	fish
PCV ($l\,l^{-1}$)	0.265	0.265	0.286	0.263	0.019	ns	ns

Key ns = non significant (P>0.05).

3.3.4.3 Teladorsagia circumcincta *L_3-specific immunoglobulin levels*

i) Immunoglobulin G (IgG)

Inoculation of larvae in week 1 *post-partum* resulted in an increase in IgG levels for all four treatment groups (Figure 3.2a). The overall average IgG level increased to 0.52 optical $density_{492}$ units (OD_{492} units) (a 2-fold increase) from 0.27 OD_{492} units pre-challenge, with a further increase to 0.8 OD_{492} units three weeks after challenge. When challenge ceased in

week 8 *post-partum* OD began to decrease. There were no significant differences in the ewe IgG levels between the four treatment groups ($P > 0.05$).

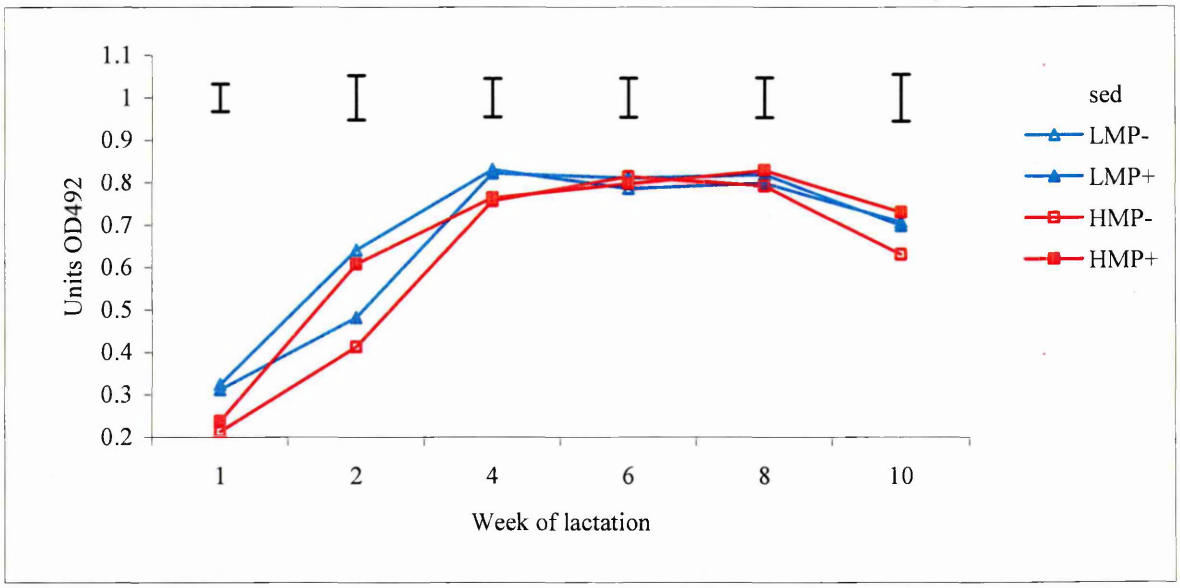


Figure 3.3 *The effect of increased MP supply and fish oil supplementation on mean ewe sera* T. circumcincta *L_3 specific immunoglobulin G levels against (OD_{492} units)*

ii) Immunoglobulin A

There was a 1.6 fold increase in serum IgA levels, of 0.32 OD_{492} units to 0.84 OD_{492} units, apparent within one week of challenge (Figure 3.2b). The IgA levels remained close to 0.84 OD_{492} units through until inoculation ceased. After challenge stopped there was a 0.8 fold decrease in OD from an overall average of 0.73 OD_{492} units to 0.4 OD_{492} units within two weeks. There were no significant differences in OD units in the sera of the ewes between the treatment groups ($P > 0.05$).

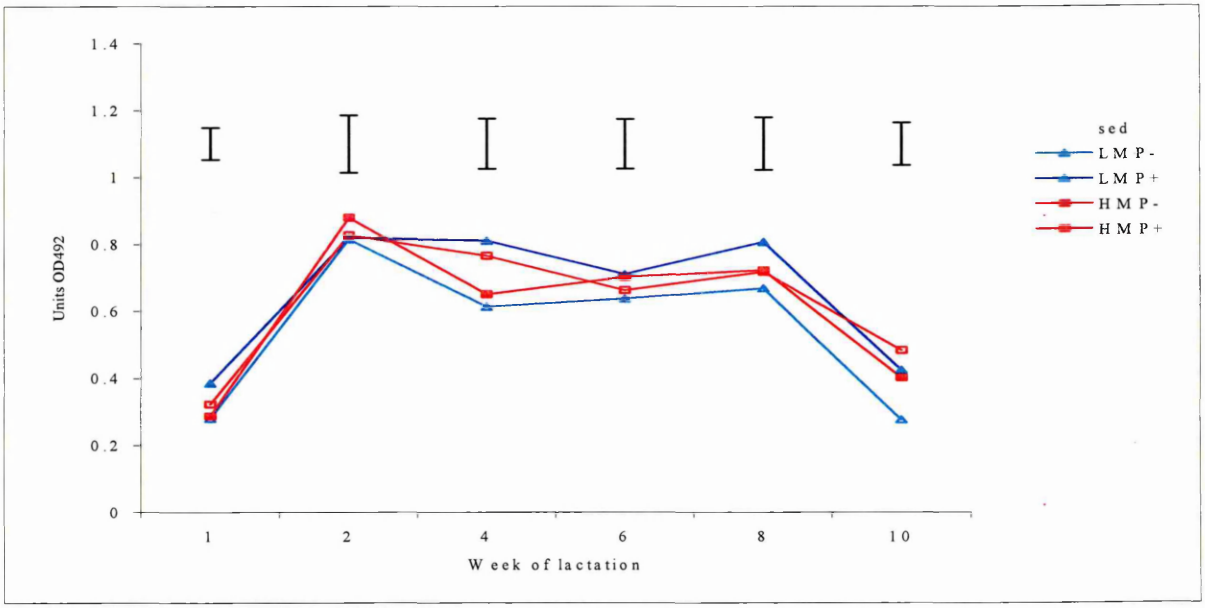


Figure 3.4 *The effect of increased MP supply and fish oil supplementation on mean ewe sera* T. circumcincta *L_3 specific immunoglobulin A levels against (OD_{492} units)*

iii) Immunoglobulin E

There were no significant differences ($P > 0.05$) in OD units for IgE in the ewe sera of the ewes in the different treatment groups, and no evidence of an effect of challenge with larvae on the IgE concentration (Figure 3.2c)

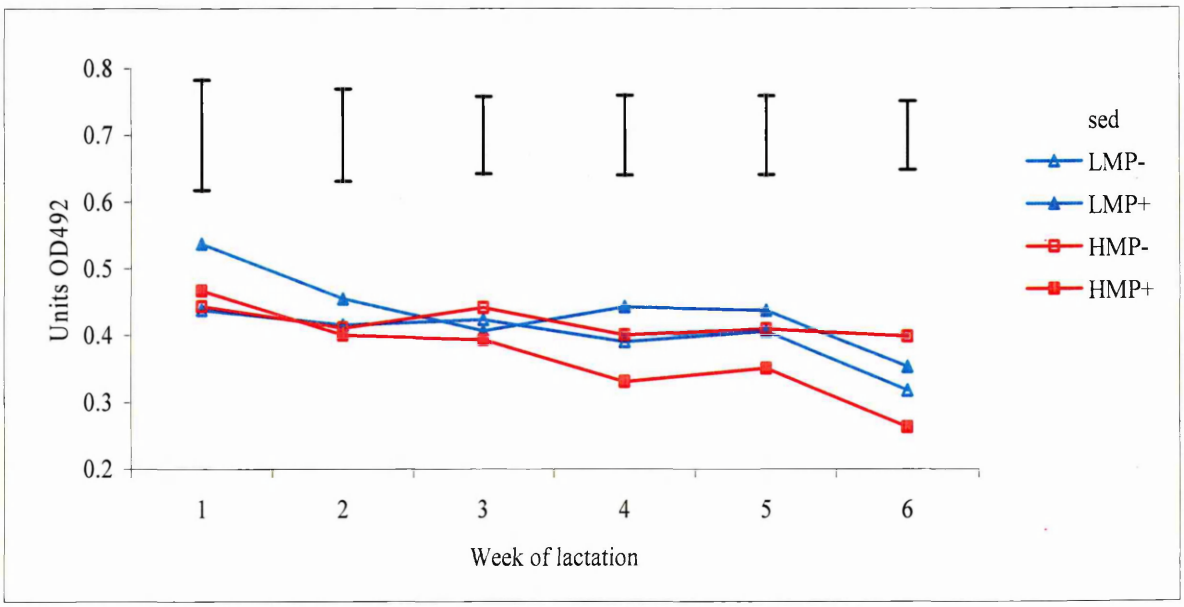


Figure 3.5 *The effect of increased MP supply and fish oil supplementation* on *mean ewe sera* T. circumcincta *L_3 specific immunoglobulin E levels against (OD_{492} units)*

3.3.5 Blood metabolites

3.3.5.1 *Plasma albumin concentration*

Prior to the experiment start the ewes assigned to the HMP dietary treatment had higher (P <0.05) plasma albumin concentrations than LMP ewes (Table 3.10). The ewes fed the HMP dietary treatments with or without fish oil continued with significantly higher plasma albumin concentration (P <0.001) until the end of challenge in week 8 *post-partum.* Throughout the experiment all the ewes were within the normal ovine range for albumin levels of between 24-30 g l^{-1}.

3.3.5.2. *Plasma urea concentration*

Before commencing the dietary treatments the ewes had an overall average plasma urea concentration of 4.4 mmol l^{-1} (Table 3.10). During the treatment period the ewes on the HMP dietary treatment had higher (P <0.001) plasma urea concentrations than the ewes on the LMP dietary treatment. Both the HMP and the LMP treatment ewes had plasma urea concentrations higher than the normal ovine range of 2.6 to 6.6 mmol l^{-1}.

3.3.5.3. *Plasma total protein concentration*

Fish oil had a significant effect on plasma protein concentration *pre-partum*; ewes fed fish oil had lower (P <0.05) protein concentrations (average 59g l^{-1}) than those fed the dietary treatments without fish oil (average 60g l^{-1}) (Table 3.10).

3.3.5.4. *Plasma β – Hydroxybutyrate (βHB) concentration*

Ewes fed the HMP treatment had higher (P<0.001) plasma βHB concentrations *post-partum* than the ewes on the LMP dietary treatments. There were no differences between the treatments *pre-partum* (Table 3.10).

3.3.5.5. *Plasma glucose concentration*

The ewes on the fish oil dietary treatments had lower plasma glucose levels (P <0.05) than the ewes on the non fish oil treatments, *pre-partum* only.

Table 3.10 *The effect of increased MP supply and fish oil supplementation on the mean ewe plasma blood metabolites* pre- *and* post-partum.

	Mean pre-treat	LMP- fish oil	LMP + fish oil	HMP - fish oil	HMP + fish oil	s.e.d.	MP	Fish
Mean metabolites *pre-partum*								
Albumin (g l-1)	*26.9*	25.8	25.2	28.4	27.8	0.914	***	ns
Urea (mmol l-1)	*4.40*	5.76	6.63	8.82	9.25	0.631	***	ns
Protein (g l-1)	*61.8*	62.6	57.3	61.4	60.7	1.799	ns	*
βHB (mmol l-1)	*0.45*	0.83	0.71	0.86	0.9	0.11	ns	ns
Glucose (mmol l-1)	*2.27*	3.08	2.85	3.2	2.88	0.188	ns	*
Mean metabolites *post-partum*								
Albumin (g l-1)		26.7	26.5	30.1	29.2	1.078	***	ns
Urea (mmol l-1)		8.6	8.7	12.1	12	0.485	***	ns
Protein (g l-1)		65.5	63.5	65.3	65.8	1.491	ns	ns
βHB (mmol l-1)		0.68	0.68	0.94	0.91	0.084	***	ns
Glucose (mmol l-1)		3.14	3.35	3.19	3.21	0.117	ns	ns

*Key ns = non significant (P>0.05), *P<0.05, **P<0.01, *** P<0.001.*

3.3.6. Milk production and composition

Ewes offered the HMP treatments produced higher yields than the ewes offered the LMP treatments with means of 1560 and 1055ml day^{-1} for HMP and LMP respectively (P < 0.01) (Table 3.11). The increase in yield due to higher MP supply increased the total (P < 0.05) milk fat, lactose, protein and solids-non-fat (SNF) produced per day. There were no differences between the treatments in the concentration of fat, SNF, lactose or protein (g kg^{-1}).

Table 3.11 *The effect of increased MP supply and fish oil supplementation on the mean ewe milk parameters.*

	LMP – fish oil	LMP + fish oil	HMP – fish oil	HMP + fish oil	s.e.d.	MP	Fish oil
Yield (ml)	1067	1044	1546	1574	212.6	**	ns
Protein (g kg^{-1})	40.3	40.8	40.0	40.4	1.40	ns	ns
Protein (g day^{-1})	42.6	42.1	60.9	62.7	7.65	**	ns
Fat (g kg^{-1})	76.8	72.6	72.6	66.1	4.68	ns	ns
Fat (g day^{-1})	82.1	75.6	110	103	14.59	*	ns
Lactose (g kg^{-1})	45.8	44.0	44.5	45.7	1.33	ns	ns
Lactose (g day^{-1})	49.2	46.1	70.5	72.3	10.46	**	ns
SNF (g kg^{-1})	86.5	85.5	85.2	87.0	1.45	ns	ns
SNF (g day^{-1})	92.4	88.9	132.9	136.7	18.44	**	ns

*Key ns = non significant (P>0.05), *P<0.05, **P<0.01.*

3.4 *Discussion*

The results obtained suggested that MP and fish oil supplementation had no effect on the faecal nematode egg output of periparturient ewes. The FECs were low from ewes on all the treatments throughout the experiment and this may account for the lack of significant results from dietary manipulation.

3.4.1. Effect of dietary treatments on FECs

The PPR period can be relatively short in well-fed sheep indoors and prolonged in sheep at pasture, this suggesting that the PPR has a nutritional basis (McAnulty *et al.*, 2001). However there was no effect of MP or fish oil on the FECs from the ewes in this experiment. It may be possible that all four of the dietary treatments provided sufficient nutrients to reduce faecal egg output, with evidence to support this being that the ewes gained between 6g and 1g per day live weight (LW) for the LMP and HMP treatments respectively during lactation despite the expectation of LW loss during lactation. Also, the increase in FECs after the treatments were terminated in week nine of lactation lends support to this hypothesis; however the complication of the termination of challenge, milking and the dietary change at that time does not enable a conclusion that all four treatments reduced FECs.

Prior to challenge in week one of lactation, the FECs were higher than during the challenge period, suggesting that a natural infection was already present. These eggs were perhaps produced by benzimidazole resistant nematodes. Because the FECs from the natural burden were high prior to artificial challenge it suggests that it was not the dietary treatments that produced the low FECs during the challenge and lactation period.

It was hypothesised that fatty acids may influence immunity as many membrane functions of lymphocytes are determined by the fatty acid composition of the phospholipids and these fatty acid profiles may be altered by diet (Johnston, 1988). However, the supplementation of the ewe diets with fish oil - n-3 polyunsaturated fatty acids (n-3 PUFA), did not reduce FECs. This is in agreement with Muturi (2003) who also found that oil supplementation did not reduce FECs or worm burden. Muturi (2003) suggested that there was an influence of fish oil on immunity to nematode infection, with calves offered fish oil had lower numbers of mucosal mast cells and eosinophils than those not offered fish oil, although whether this was a beneficial influence was not concluded. Indeed, Wu and Meydani (1998) have suggested that increased EPA-derived eicosanoids can contribute to immuno-suppression involving suppression of helper T cells in humans and animals.

There may be no beneficial influence of fish oil on abomasal dwelling nematodes or PUFAs may need to have direct contact with nematodes in the abomasum. The PUFAs available in the current experiment may not have avoided biohydrogenation in the rumen as unsaturated fatty acids have a relatively short half-life in ruminal contents because they are rapidly hydrogenated by microbes to more saturated end-products (Jenkins, 1993). Chikunya *et al.* (2004) found that greater than 90% of n-3 PUFAs in fish oil were susceptible to biohydrogenation by rumen micro-organisms. This contrasts Palmquist and Kinsey (1994) and Ashes *et al.* (1992) who claimed that EPA and DHA may possess a natural protection from biohydrogenation. Fish meal used in previous studies by Donaldson *et al.* (1998; 2001) may have not only offered ruminal protection for protein but also protected the n-3 PUFAs from biohydrogenation in the rumen.

The levels of EPA and DHA offered in the experimental diets were similar levels fed to sheep by Wachira *et al.* (2002) who fed between 3 and 5.7g kg^{-1}, 1.6 and 3.3g kg^{-1} EPA and DHA respectively, and Chikunya *et al.* (2004) who fed 2.6 and 1.4g day^{-1} of EPA and DHA respectively. The current experimental diets offered 0.26, 1.85, 0.41 and 1.29g kg^{-1} EPA and 0.25, 0.51, 0.19 and 0.27g kg^{-1} DHA for the LMP-, LMP+, HMP- and HMP+ fish oil diets respectively which would have been higher than Chikunya *et al.* (2004) had offered per day considering the ewes consumed in the region of 2-2.5kg DM per day during lactation. There may not have been a significant enough difference in DHA concentrations between the diets although there was a difference in the levels of EPA fed to the ewes in the current experiment, perhaps the lack of difference in DHA offered lead to the lack of differences between treatment groups in FECs.

3.4.2. Effect of larval challenge on FECs

The most plausible explanation for the low FECs throughout the challenge period, which occurred within a week of challenge, and the increase in FECs after challenge ceased could have been that the increased host defences against the incoming larvae reduced the fecundity of the adult worms. Indeed, Stear *et al.* (1995; 1999) suggested that worm numbers and lengths (which are positively correlated with numbers of eggs *in utero*), were regulated by an immediate hypersensitivity reaction associated with an increased local IgA response to fourth stage larvae. An alternative explanation could be a density dependant competition from the incoming larvae. Increases in worm burden are often associated with reductions in mean worm length and fecundity (Sykes and Poppi, 1982; Poulin, 1998; Dezfuli *et al.*, 2002).

In the Friesland ewes there was an increase in circulating eosinophils, IgA and IgG soon after challenge commenced. Both IgA and IgG remained at high levels until the termination of challenge showing a reaction against the incoming larvae and these are known to reduce the fecundity of adult nematodes also (Stear *et al.*, 1995). As there were

no differences between treatments in peripheral eosinophil, IgA and IgG concentrations this could confirm that all the dietary treatments provided sufficient nutrients to mount an immune response.

T. circumcincta was chosen as it is the dominant nematode in sheep in cool, temperate areas such as the UK (Stear *et al.*, 2002). However, the challenge rate of 14,000 larvae per week may have been too low and not an accurate example of a natural exposure to nematodes. Brunsdon (1982) suggested that larval intake per day at pasture, ranged between 1,000 and 5,000 larvae day^{-1}. Donaldson (1998) described evidence of 3,000 larvae kg^{-1} herbage in mid-winter and Vlassoff (1982) provided an estimate of about 500 larvae kg^{-1} herbage available to the ewes in spring. It would be reasonable, taking into consideration the variation possible in pasture larval concentrations, and assuming ewes consumed approximately 1.2kg herbage ewe^{-1}, that an infection rate of 2,000 larvae day^{-1} would be representative of a natural exposure to larvae at pasture in spring. It is difficult to estimate a number of larvae that may be ingested by the average ewe at pasture day^{-1} as there are many factors such as climate (season), herbage height and the fact that faecal contamination is often highly clumped and the infectivity of a pasture may vary considerably from area to area (Vlassoff, 1982). Depending on developmental success the pasture larval contamination may vary between a few hundred and several millions of larvae day^{-1} (Vlassoff, 1982). Another difficulty in estimating larval intake is that ewes can consume varying amounts of herbage; indeed Gibb *et al.* (1981) noted a peak intake of 3.75kg organic matter day^{-1} by week 3 of lactation.

Although FECs are often used as an indication of the size of nematode infection in epidemiological studies there are limitations in associating greater egg counts with greater worm establishment (Mansour *et al.,* 1991). Faecal egg counts are susceptible to variation in feed residues passing down the tract (Sykes and Poppi, 1982). To reduce the variation, all the FECs were performed at the same time of day to avoid a variation in feed levels; however the low FECs may have been due to a high quantity of food passage at that time.

A parasitological explanation for the lack of differences in FECs between the treatment groups could be that the nematode burdens were similar across all treatment groups, although worm burden analysis was not performed. According to Barnes & Dobson (1993), Eysker & Ploeger (2000) and Mansour *et al.* (1991), FECs may not reflect the actual worm burden, and the ewes could, therefore have been carrying a larger worm burden than the FECs represented. However, in respect to reducing the pasture contamination from the periparturient ewe and the subsequent infection of the lambs, the aim of nutrient supplementation would be to reduce FECs, whether by reducing adult nematode fecundity or by reducing nematode burden.

3.4.3. Effect of dietary treatments on ewe haematological and immunological parameters

Eosinophils, attracted to sites of helminth invasion by chemotactic factors released by degranulating mast cells (globule leukocytes), are mobilised in large numbers into the circulation from the bone marrow (Tizard, 1992). Previous workers have suggested that an increased supply of MP may 'enhance the expression of immunity' of parasitized growing lambs (Bown, *et al.*, 1991; van Houtert, *et al.*, 1995). As many components of the immune system, such as eosinophils, mast cells and globule leukocytes, are proteinaceous in nature it is possible that they draw on MP resources (MacRae, 1993; Coop and Holmes, 1996). By increasing the MP supply to the ewes in this experiment, it was presumed that they would have shown a greater rise in eosinophil count than the ewes on the LMP dietary treatments, which did not occur. The HMP ewes produced only a 6.7-fold increase in eosinophils compared with the LMP ewes, which produced a 17-fold increase in response to challenge in week 3 of lactation. The ewes offered the fish oil supplemented treatments showed a greater increase in eosinophils in comparison to unsupplemented ewes, although this effect was not significant. This could possibly indicate that it was a fatty acid component and not

the MP supply in fishmeal that played a part in the reduction of the PPR found in previous work by Donaldson *et al.* (1998; 2001).

There were no differences between the dietary treatment groups for anti- *T.circumcincta* IgG titres, this is in agreement with Coop and Holmes (1996) who noted that the level of specific and non-specific circulating antibodies were generally not affected by protein nutrition, and contrasts with Israf *et al.* (1996) who found that fish meal supplementation of *Nematodirus battus* infected lambs increased anti-worm IgG titres. Immunoglobulin G levels increased up until week four after inoculation in week one, before plateauing until the end of inoculation in week eight. Immunoglobulin G plays a part in the immunity to parasites by being attracted to the site of invasion by IgE-mediated responses such as the release of potent vasoactive molecules. The IgG then triggers an acute inflammatory response (Tizard, 1996). The results of the IgG ELISA suggest that they had been involved in the immunity against the incoming larvae, but the different treatments had not changed the magnitude of their reaction.

Immunoglobulin A is found on the gut mucosa and the most important function of this molecule is to prevent the adherence of pathogens to epithelial surfaces (Tizard, 1996). For this reason very little IgA would be expected in the blood stream (Huntley *et al.*, 1998). Stear *et al.* (1999) suggested that IgA appears to be the major mechanism regulating worm length, and consequently their fecundity. There were no differences between treatment groups in serum IgA concentration; however there was a reaction to challenge with larvae in the form of a 1.5-fold increase in IgA in the blood circulation by week two of challenge. The IgA remained at the higher levels until challenge ceased in week eight. Decreased fecundity of the nematodes present prior to challenge with infective stage larvae may have been due to an increased IgA response to the fourth stage larvae (Stear *et al.*, 1995) which could explain the decrease in FECs by week 2 of lactation.

Serum IgE concentration decreased after challenge commenced. Immunoglobulin E is the most significant isotype involved in resistance to helminths (Tizard, 1992). However,

Tizard (2000) suggested that only when invading helminths evade the initial IgA protection and gain access to the tissues does the IgE-mediated response occur. These results therefore suggest that the IgA response had been sufficient in guarding against the invading larvae at the mucosal level.

Overall, the dietary treatments had no effects on any of the immunoglobulins, suggesting that although they are proteinaceous molecules as stated by MacRae (1993) and Coop and Holmes (1996), simply increasing the MP supply to the ewes does not result in a greater increase in immunoglobulin concentration than a lower MP supply. Fish oil may have had no effect on immunoglobulins as Wu and Meydani (1998) have suggested that PUFAs may have a immunosuppressive action.

3.4.4. Effect of dietary treatments on ewe condition score

Russel (1984) suggested that body condition scoring would be a more useful means of assessing the adequacy of nutrition than LW. Live weight is a poor indicator of ewe nutritional status during lactation as there is an increase of water content due to body fat mobilisation and an increase in the weight of digesta in the alimentary tract caused by an increased voluntary feed intake during early lactation (Treacher and Caja, 2002). There were no differences in CS change between the treatment groups throughout the experimental period and the slight loss of CS could be attributed to the nematode burden which is known to reduce ewe condition (Leyva, 1982). The low CS of the ewes in the current experiment, however, could be attributed to the breed as according to Mills (1989) many dairy ewes have CS of less than 2.5. Indeed, the Friesland ewes studied by Wilkinson *et al.* (2000) had a mean CS of 1.5.

It is likely that the lack of differences in CS between the low and high MP supplied ewes was due to the low CS throughout the experiment as Jones and Garnsworthy (1988) found that increasing UDP to thin cows had no effect on CS, yet in fat cows it lead to an increase in mobilisation of body fat reserves. Additionally, the mobilisation of body fat reserves

may only be exploited if body tissue loss is not physiologically detrimental to the animal (Wilkinson *et al.*, 2000).

3.4.5. Effect of dietary treatments on ewe plasma metabolites

3.4.5.1. *Indicators of energy status*

Plasma glucose levels were measured as an indicator of energy status, with values of below 3.1 mmol l^{-1} indicating an energy deficiency (Topps and Thompson, 1984). Plasma glucose can decline following under-feeding or fasting, but the extent of the decline is not usually related to the degree of under-nutrition (Topps and Thompson, 1984). Ewe plasma glucose levels in this experiment remained above the recommended 3.1 mmol l^{-1}, for all the treatment groups.

Beta-hydroxybutyrate (βHB) was measured as it is regarded as an acceptable method of assessing energy intake of ruminants as it is stable in blood and more convenient to determine than total ketones (Topps & Thompson, 1984). Excess acetate from the rumen, excess body fat mobilisation or too little glucose can cause an increase in ketones (Topps & Thompson, 1984). Plasma βHB levels in this experiment were similar between treatment groups until after parturition. *Post-partum,* the HMP treatment ewes had higher βHB concentrations ($P<0.05$) than the LMP treatment ewes. Although mean values did not exceed the normal acceptable range of 0.6-1.2 mmol l^{-1} (Russel, 1984), some individuals in the HMP diet groups were, this suggested that the energy intake may have been low.

In view of plasma βHB levels being affected by the time of sampling (Topps & Thompson, 1984), the ewes may have had higher βHB levels and therefore even lower glucose levels prior to the morning feed as opposed to only two hours after feeding. Sampling prior to the morning feed may have produced more accurate results of nutrition adequacy as suggested by Hussain *et al.* (1996), as this would be the time when the absorption of nutrients from digestive tract was at a minimum. There may have been significantly higher plasma βHB

concentrations in the HMP treated ewes at this time which may have confirmed fears that the diet was low in energy supply for dairy ewes. If plasma βHB levels had been high then this may have explained the low milk yields as limited energy supply can be a limit milk production (Cannas, 2002a).

3.3.5.2 *Indicators of protein status*

Plasma albumin levels were measured as a reflection of the animal's ability to synthesize and store protein, and a low value may have indicated an insufficiency of protein and/or energy supply over an extended period (Topps and Thompson, 1984). The ewes on the HMP treatment had higher albumin levels ($P<0.05$) than ewes on the LMP treatment throughout the experimental period (26 and 29 g l^{-1} for the LMP and HMP treatment groups respectively). These results reflect that the protein level was higher in the HMP treatments.

Teladorsagia circumcincta can cause a loss of albumin into the gut and cause hypoalbuminaemia (Holmes and MacLean, 1971, Topps and Thompson, 1984). Any difference in albumin level could, therefore, be attributed to different parasite burdens as well as differences in protein in the diet. The differences in albumin between the treatments in this experiment were more likely to be due to the different protein levels fed as opposed to the parasite burden.

Plasma urea is a measure of the excess ammonia produced in the rumen, as a result of protein breakdown, which is carried in the blood and converted into urea by the liver and later excreted by the kidneys (Topps and Thompson, 1984). The ewes on both the HMP and LMP treatments had very high plasma urea concentrations, although the LMP diets took longer to exceed the recommended range of 2.6-6.6 mmol l^{-1}. By the end of the experiment, the ewes on the HMP treatments had urea levels in excess of 13 mmol l^{-1}.

High plasma urea concentrations which are accompanied by hypoalbuminaemia could indicate a parasitic or pathogenic infection of the liver (Topps and Thompson, 1984).

The high plasma urea levels were probably indicative of the different levels of protein fed to the ewes as urea levels can increase in accordance with protein levels fed (Choung *et al.*, 1990), or could suggest that the ratio of protein to energy in the rumen was not synchronous (Sinclair, 1993, Witt *et al.*, 1999). If rumen micro-organisms do not have sufficient energy to enable the ammonia produced in the rumen to be absorbed into microbial protein, ammonia will be lost from the rumen and blood levels will increase, at an energy cost to the animal (McDonald *et al.,* 1995).

In circumstances where the intake of rumen-degradable protein remains relatively constant, plasma urea may be inversely related to the intake of dietary energy (Topps & Thompson, 1984) and the high urea levels could have been indicative of a deficiency in energy supply. The ewes offered the HMP treatments had higher blood urea (P <0.001) and βHB (during lactation only $P < 0.001$) concentrations which could represent a greater deficiency in dietary energy intake than the ewes offered the LMP treatments. The HMP treatments may not have had enough energy in relation to protein supply and therefore the ewes on those diets may not have been able to utilise all the protein offered.

Fish oil supplementation had no effects on blood urea, albumin, glucose or βHB concentrations and neither fish oil nor MP level had any effect on blood total protein levels.

3.4.6. Milk yield and its effect on FECs

The objective of using dairy ewes was to provide data from animals under a higher production pressure than ewes suckling twins. McDonald *et al.,* (1995) suggested that dairy ewes were under a high production pressure, particularly within the first two to three weeks of lactation. Despite this, the ewes produced only an average of 1.0 and 1.5 kg day^{-1}

for the LMP and HMP treatments respectively. This is low when compared to that produced by Chikunya *et al.* (2002) in the study of Frieslands fed *ad-libitum*, which produced an average of, just under 2.0 kg day^{-1}, but above that in work by Wilkinson *et al.* (2000) who reported yields of between 1.5 and 1.1 kg day^{-1} using grazed Friesland ewes provided with concentrates differing in ERDP and DUP levels. The ewes in the current experiment fed the HMP treatment, produced higher milk yields ($P < 0.05$) than the LMP treated ewes. This was anticipated as increasing the metabolisable protein supply has been shown to improve milk yield in dairy cows (Oldham, 1984), sheep (Treacher and Caja, 2002; Brocquier and Caja, 2004) and goats (Chartier *et al.,* 2000). There may have been a shortage in energy supply as Cant *et al.* (2001) suggested that it was ME not MP that limited dairy ewe performance; however, Treacher and Caja (2002) suggested that at a particular level of ME intake there was a critical protein intake below which yield decreased.

The low milk yields may have been the result of the rupture of the maternal bond or the lack of suckling stimuli and the subsequent reduction of frequency of udder emptying (Labussière, 1988; Mills, 1989; Marnet *et al.*, 1998; McKusick *et al.*, 2002). Weaning within 4 days of birth reduces the 'stresses of weaning and subsequent reduced milk yield compared to weaning at 42 days (Labussière, 1988). However, weaning at around 30 to 42 days often coincides with the peak of lactation and therefore a loss in milk yield would be apparent (Cant *et al.*, 2001). Therefore, the stress of weaning at 72 hours may have been sufficient to cause a substantial decrease in milk yield. However, weaning maybe discounted as a major contributing factor to the low yields as research undertaken by Chikunya *et al.* (2002) using the same flock and weaning method had higher mean milk yields throughout the treatments, with the key difference between experiments being that ewes were fed *ad-libitum.*

Leyva *et al.* (1982) found that infection with *T. circumcincta* could reduce milk production. However, because there was no non-parasitised treatment in the current

experiment it is not possible to define whether the low yields were due to nematode burden or not.

Inadequate feeding of the lactating dairy ewe may reduce both the daily milk production and the length of the lactation (Cannas, 2002a). Indeed, Cannas (2002a) suggested that AFRC (1993) calculations for lactating sheep were developed for the meat or wool breeds of sheep and not dairy ewes and that AFRC (1993) rations tend to underestimate the feed requirements of lactating dairy ewes. When comparing the diets fed in this experiment to requirements suggested by Cannas (2002) of 166g kg^{-1} DM CP for the average 65kg LW ewe producing 2.5litres milk day^{-1}, the protein requirements were above requirements for the HMP treatments at 180g kg^{-1} DM CP and lower for the LMP treatments at 140g kg^{-1} DM CP. The feeding level of 1.8kg DM day^{-1} during lactation was sufficient for lactating ewes producing 2.5 litres milk day^{-1} according to Cannas, (2002); however Friesland dairy ewes when fed *ad-libitum* in the work of Chikunya *et al.* (2002) had a daily DM intake on average of 2.7kg day^{-1} and Gibb *et al.* (1981) noted a peak intake of 3.75kg organic matter day^{-1} by week 3 when lactating ewes grazed a daily herbage allowance of up to 60g DM kg^{-1} LW of the ewe. Peart (1968) and McDonald *et al.* (1995) suggested that even a relatively small restriction of intake can depress milk production which could explain the lower yields observed here. Despite this it is not obvious whether it was diet, milking method or nematode challenge which caused the low yields in this experiment.

3.4.7. Effect of dietary treatments on milk composition

Milk protein decreased within one week of lactation from 45.5g kg^{-1} to approximately 39.5g kg^{-1} and remained constant thereafter. Milk lactose and fat concentration remained constant at around 40.3g kg^{-1} and 72.3g kg^{-1} respectively. Although the differences in milk fat content were not significant, ewes offered the HMP diets tended to have lower fat concentrations than the ewes on the LMP treatments. This is likely to be due to dilution from the significantly higher yields that the HMP treated ewes produced. As described

earlier under-nutrition can lead to a decrease in milk yield, which is often accompanied by a decrease in protein content with an increase in fat content, due to an increase in blood free fatty acids as a consequence of increased body fat mobilisation (Bocquier and Caja, 2004). There may have been little influence of diet on the milk composition in these ewes as these ewes had a low mean body CS of 1.5 and therefore body fat mobilisation may not have been exploited as Jones and Garnsworthy (1988) suggest that this response is not exploited if body tissue loss could be detrimental to the animal.

Milk fat yield was reduced ($P < 0.05$) by the supplementation of the diets with fish oil and this was not due to a dilution effect of increased milk yield. Chikunya *et al.* (2002) also found that fish oil resulted in a significant reduction in milk fat concentration. Sinclair *et al.* (2005) suggest this may have be due to the production of the rumen biohydrogenation intermediate - *trans*-10, *cis*-12 conjugated linoleic acid (CLA) which is known as a potent inhibitor of milk fat synthesis in lactating sheep. However, CLA is a positional isomer of the linoleic acid (C18:2 n-6; Bessa *et al.*, 2000). The lactation diets fed during the current experiment contained similar amounts of linoleic acid of 8.86, 7.84, 7.86 and 7.29g kg^{-1} DM for the LMP-, LMP+, HMP- and HMP+ fish oil, respectively. Donovan *et al.* (2000) reported similar findings of increased CLA production when cattle are fed fish oil and proposed that something in fish oil may serve as a ruminal modifier to stimulate additional synthesis of CLA from linoleic acid already present in other dietary ingredients. This would explain the lower milk fat yields produced by the ewes offered the fish oil diets.

It would be difficult to estimate whether or not milk composition overall treatments had been affected by parasites as there were no non-challenged treatments and McDonald *et al.* (1995) suggested that stage of lactation and the breed of the ewe could also affect ewe milk composition. Factors such as sampling techniques, stage of lactation and milking intervals all affect composition, thus, published figures for average fat and protein contents vary from 50g kg^{-1} to 100g kg^{-1} and from 40g kg^{-1} to 70g kg^{-1}, respectively (McDonald *et al.*, 1995; Table 3.12).

Table 3.12 *The mean ewe milk composition of sheep (MacDonald et al., 1995; Treacher and Caja, 2002) compared to the composition from the experimental ewes.*

($g\ kg^{-1}$ whole milk)	Levels in Treacher and Caja, (2002)	Levels in McDonald *et al.* (1995)	Experiment levels
SNF	115	119	96
Fat	71	74	72
Protein	57	55	40
Lactose	48	48	45

3.5. *Conclusions*

In conclusion, a combination of competition from existing larvae and increased immunity against the incoming larvae, helped by the sufficient resources from all the dietary treatments, which was most possibly due to the reduced milk yields caused by the lack of lamb suckling stimuli and/or low milking frequency, would be the most likely explanation of the lack of differences in FECs between treatment groups.

CHAPTER FOUR – EXPERIMENT TWO

THE EFFECTS OF METABOLISABLE PROTEIN SUPPLY ON THE PERIPARTURIENT RISE IN FAECAL EGG COUNTS IN DAIRY EWES

4.1 *Introduction*

In the previous experiment, there was little evidence to suggest that the ewes had experienced a PPR or that there was any link between nutrition and susceptibility to nematodes. These findings are in contrast to several authors that have described a link, predominantly in respect to nutritional protein status (Kambara *et al.*, 1993; Coop *et al.*, 1995; van Houtert *et al.*, 1995; Wallace *et al.*, 1999; Donaldson *et al.*, 2001; Houdijk *et al.*, 2000; 2001c; 2003). However, none of the aforementioned authors have worked with machine-milked dairy ewes.

The current experiment aims to expand on the previous results and study the effects of vegetable sourced MP and fish oil on nematodes in the periparturient machine-milked dairy ewe. This experiment aims to differentiate between whether nematode burden or the frequency of udder emptying had reduced milk yields whilst still aiming to distinguish if a greater level of vegetable-sourced MP is needed to reduce FECs in dairy ewes. It is likely that milking frequency would have a critical effect on milk yields in early lactation (McKusick *et al.*, 2001), so milking frequency was increased to 3 times a day.

Due to evidence that protein can successfully reduce the FECs of periparturient ewes (Donaldson *et al.*, 2001) whilst studies investigating fatty acids (Muturi, 2003) are less clear, it was decided to omit fish oil from this experiment.

4.2 *Material and methods*

4.2.1 Experimental design and animals

Fifty pedigree Friesland ewes were oestrus-synchronised (section 3.2.1) to result in a mean parturition date of 8th April 2002. The ewes were ultra-sound scanned at 71 days of gestation. At 8 weeks prior to parturition the ewes were dosed with Panacur™, (Hoechst Roussel Vet Ltd., Milton Keynes, UK) at the manufacturer's recommended dose (5mg kg^{-1} live weight), and immediately group-housed. The ewes were then offered wheat straw *ad-libitum* and remained group-housed until the start of the experiment at five weeks prior to parturition.

Faecal egg counts were performed one week after dosing. The ewes continued to expel nematode eggs despite the treatment and were therefore dosed again with Levacur SC™, (Hoechst Roussel Vet Ltd., Milton Keynes, UK,) at the manufacturer's recommended dose (7.5 mg kg^{-1} live weight) at five weeks prior to parturition. The ewes were also subcutaneously injected with Heptavac-P plus™ vaccine, (section 3.2.1.) at 4 weeks prior to parturition.

Thirty-two ewes were selected for use in the experiment (20 twin-bearing ewes, 8 triplet-bearing ewes and 4 single-lamb bearing) of varying ages ranging from 2 to 9 years. The ewes were blocked according to foetal number, live weight (LW), condition score (CS), age and previous lactation yields and allocated to one of 4 treatment diets (n=8, consisting of 1 single, 2 triplet and 5 twin bearing ewes). The experimental design was a 2 x 2 factorial (n = 8) comprising of two dietary treatments and two parasitological treatments. The diets differed in levels of metabolisable protein (MP) supply with a basal MP (BMP) treatment (0.99 x requirements [AFRC, 1993]) and a high MP (HMP) treatment (1.74 x requirements [AFRC, 1993]). The treatment groups comprised of a basal MP uninfected (BMP-N), high MP uninfected (HMP-N), basal MP infected with *T. circumcincta* larvae (BMP-I) and high MP infected with *T. circumcincta* larvae (HMP-I). The ewes were housed as in experiment 1 (section 3.2.1.).

Table 4.1. *Dietary composition of feed offered* pre- *and* post-partum *(g kg^{-1} DM).*

	Pre-partum diet		*Post-partum* diet	
	Basal MP	High MP	Basal MP	High MP
Composition (g kg^{-1} DM)				
Ground barley	332	202	271	118
Molassed sugar beet pulp	179	114	146	62.4
Lactamine™ *	0	198	0	240
Fat prills	24	24	24	24
Rape seed meal	18	18	72	72
Soya bean meal	0	0	42	42
Urea	10	8	9	6
Minerals & Vitamins	18	18	18	18
Molasses	18	18	18	18
Hay	400	400	400	400
Total	1000	1000	1000	1000
Predicted diet composition				
ME MJ kg^{-1} DM	11.6	11.4	11.7	11.4
CP g kg^{-1} DM	148	223	176	265
ERDP 5[1]	140	140	-	-
DUP 5[1]	31	126	-	-
ERDP 8[2]	-	-	306	310
DUP 8[2]	-	-	104	337
ERDP/FME ratio	10.0	10.4	10.9	11.4
MP g kg^{-1} DM	122	216	300	528
Determined composition (concentrate only)				
Dry matter (g kg^{-1})	851	859	861	871
Crude protein (g kg^{-1} DM)	147	223	167	339
Ash (g kg^{-1} DM)	61	70	64	81
Ether extract (g kg^{-1} DM)	43	48	36	65
Neutral detergent fibre (g kg^{-1} DM)	188	213	209	209

[1] calculated at a rumen solid phase outflow rate of 0.05 h^{-1} (g kg^{-1}) (AFRC, 1993)
[2] calculated at a rumen solid phase outflow rate of 0.08 h^{-1} (g kg^{-1}) (AFRC, 1993)
* Lactamine™, a metabolisable protein source of sopralin and methionine, (Trouw Nutrition, Northwich, UK.).

The ewes were offered the *pre-partum* diets from 5 weeks prior to parturition. *Pre-partum* feeding levels were restricted to levels recommended by AFRC, 1993 according to predicted litter size and weekly live weight on the assumption that they would gain 0.075kg LW day^{-1}. The *post-partum* diets were offered from week 1 through to slaughter at either weeks 6 and 7 or 10 and 11 *post-partum*. *Post-partum* diets were fed at a restricted level, on the assumption that the ewes were producing 2.5 litres of milk and losing 0.05kg day^{-1} LW (AFRC, 1993). Hay was offered separately in a ratio of 60:40, concentrate to

hay, respectively, both *pre-partum* and *post-partum* (Table 4.1). Equal amounts of ration were offered at 08.00 hours and 16.00 hours *pre-partum* and 08.00h and 15.00h *post-partum*.

Lamb birth weights were recorded when dry and within 6 hours of birth. The lambs were then weaned within 2 days of birth and reared on milk replacer. The ewes were machine-milked twice a day (07.30 and 16.00 hours) for the first week and then 3 times a day from week 2 (07.30, 14.30 and 21.00 hours) until week 7 of lactation and thereafter twice a day (08.00 and 16.00 hours). Eight ewes were slaughtered at one of the 4 slaughter dates for collection of the abomasal contents and mucosa (sections 2.4.3 & 2.4.4).

4.2.2 Parasitology

4.2.2.1. *Faecal egg count reduction tests*

Faecal egg count reduction tests (FECRT) were performed prior to the start of the experiment, using a method similar to that described by Coles *et al.* (1992) except without an untreated control group. The test provides an estimate of anthelmintic efficacy by comparing worm egg counts from animals before and after treatment. Immediately prior to treatment with Panacur™, (Hoechst Roussel Vet Ltd., Milton Keynes, UK) at the manufacturer's recommended dose (5mg kg^{-1} live weight) on 15/2/02 faecal samples were taken and the FECs recorded. Ten days later (25/2/02) FECs were carried out again. After failure to abolish the presence of nematode eggs, the ewes were dosed on the 4/3/02 with Levacur SC™, (Hoechst Roussel Vet Ltd., Milton Keynes, UK,) at the manufacturer's recommended dose (7.5 mg kg^{-1} live weight) immediately after taking a pre-dosing faecal sample for FECs. Seven days later (11/3/02) FECs were carried out again.

4.2.2.2. *Larval challenge*

The two challenged groups received approximately 2000 *Teladorsagia circumcincta* (Moredun ovine anthelmintic susceptible isolates; Moredun Research Institute, Penicuik,

UK) infective larvae (L_3) per day from week 3 prior to parturition until week 6 *post-partum*. This was achieved by placing 3ml of the infective larvae in a water suspension (approximately 4,650 L_3 per 3ml) on a dampened Whatman™ 1 filter paper of 110mm diameter (Whatman International Ltd., Maidstone, UK) and administered as described in section 3.2.2.

4.2.2.3. *Pepsinogen assays*

Pepsinogen concentrations were measured using plasma recovered from whole blood collected in vacutainers containing the anticoagulant Lithium Heparin which had been stored at -20°C until analysis. The assay performed was based on a method used by Mylea and Hotson (1969) and modified to be read by a ninety-six well Microplate Reader (Biolab-Benchmark) (*See Appendix A*).

4.2.3 Sample collection

Faecal samples were obtained from all ewes once weekly as described in section 3.2.3. Sampling commenced eight weeks prior to parturition, (3 weeks prior to the start of the experiment and ceased in the week of slaughter). The faecal egg counts were determined using a modified floatation method (Christie and Jackson, 1982), in which polyallomer centrifuge tubes were used to collect the nematode eggs from the meniscus (Moredun Research Institute, Penicuik, Scotland) (section 2.4.2).

Blood samples were collected fortnightly into evacuated tubes as described in section 3.2.3. A 1ml sample of heparinised whole blood was collected for eosinophil counts and the EDTA-treated whole blood was collected for subsequent haematology analysis which was performed within 5 hours of sampling. The remaining samples were centrifuged and the plasma and serum stored at -20°C until subsequent metabolite analysis (sections 2.5 to 2.5.3).

The ewes were milked three times a day in a Fullwood automatic goat-milking parlour. Milk samples were collected weekly commencing in week 1 *post-partum* on three consecutive morning, afternoon and evening milkings and placed in 20ml capped milk tubes (Massmould, Luton, Beds. UK). The samples were frozen at -20°C until analysis (section 2.6). Milk yield was recorded at all 3 milkings and the daily milk yield calculated. Ewe LW and CS were recorded once a week on a Wednesday at 2pm (section 2.3.1). Lamb birth weight was measured as described in section 2.3.2. Samples of the treatment diets were collected weekly and stored at -20°C for analysis (section 2.1).

4.2.4. Statistical analysis

Data was tested for normality and any data found to be skewed was transformed by Log_{10} (n+1). Statistical analysis was performed on Genstat release 7.2. Lawes Agricultural Trust (2004) and analysed as a 2 x 2 factorial analysis of variance (ANOVA), with main effects of protein level (MP), infected or non-infected (Inf) and their interaction (Int). Skewed data was presented as the back-transformed-means (BTM) with the back-transformed 95% confidence intervals (CI) representing the error bars on the graphs.

4.3 *Results*

4.3.1 Sheep health

Throughout the experiment there were no clinical signs (section 3.3.1) of infection with *Teladorsagia circumcincta.* Five of the ewes had foot rot from the onset of the experiment which was treated *post-partum* (section 2.3.). Data from seven of the ewes was omitted from the statistical analysis; one ewe was not pregnant, one ewe was fed as a twin-bearing ewe but only gave birth to 1 lamb, another was fed as a single bearer and gave birth to twins. Three ewes produced no milk and lastly one of the non-inoculated ewes had extraordinarily high faecal nematode egg counts (FECs) and high worm burden compared to all the rest of the ewes.

4.3.1.1. *Ewe live weight, condition scores and lamb birth weight*

Ewes offered the high MP treatment gained more ($P < 0.05$) weight during pregnancy than those offered the basal MP treatment (Table 4.2); however, MP supply had no effect on LW during lactation. During pregnancy there was an interaction between MP supply and nematode infection on the CS of the ewes ($P < 0.05$), during lactation the ewes on the basal MP treatments (BMP-N and BMP-I) gained more condition ($P < 0.05$) than those offered the high MP treatment. The treatments had no effect on lamb birth weights ($P > 0.05$) which averaged 4.03kg.

Table 4.2. *The effect of dietary MP and nematode challenge on ewe live weight (kg), condition score and lamb birth weight (kg).*

	BMP-N	HMP-N	BMP-I	HMP-I	s.e.d.	MP	INF	Int
Pre-partum LW (kg)								
Week -5	64.2	63.3	65.7	64.7	4.06	ns	ns	ns
Week -1	76.6	78.1	77.6	79.9	4.83	ns	ns	ns
Pre-partum change	+12.4	+14.8	+11.9	+15.2	1.67	*	ns	ns
Pre-partum CS								
Week -5	1.9	1.9	1.8	1.9	0.15	ns	ns	ns
Week -1	2.1	1.9	1.8	2.3	0.14	ns	ns	**
Pre-partum change	+0.19	+0.00	+0.09	+0.43	0.141	ns	ns	*
Post-partum LW (kg)								
12 hr *post-partum*	63.1	66.7	62.3	67.7	3.94	ns	ns	ns
Wk 6 *post-partum*	63.7	67.3	65.9	64.4	3.02	ns	ns	ns
Post-partum change	+0.57	+0.60	+3.60	-3.23	2.395	ns	ns	ns
Post-partum CS								
12 hr *post-partum*	1.9	2.0	1.7	2.0	0.14	*	ns	ns
Wk 6 *post-partum*	2.1	2.1	2.1	2.1	0.13	ns	ns	ns
Post-partum change	+0.19	+0.05	+0.35	+0.04	0.129	*	ns	ns
Lamb weights (kg)								
Mean lamb bwgt	4.20	4.02	3.86	4.05	0.226	ns	ns	ns
Mean litter weights	8.80	8.47	7.94	8.52	0.634	ns	ns	ns

*Key, ns = non significant, * P < 0.05, ** P < 0.01, *** P <0.001*

4.3.2 Parasitology

4.3.2.1 *Faecal egg count reduction tests*

Prior to the administration of Panacur™ (day 0), the average FEC was 222 (range 7-1152) eggs per gram (epg) over all the ewes. By day 10, the FECs averaged 51.4 (range 2-480) epg therefore the treatment had reduced the FECs by only 76.8%. Immediately prior to the administration of Levacur SC™ (day 19), the average FEC was 104.7 (range 0-867) epg. Seven days after Levacur SC™ treatment (day 26) the average FEC was 9.4 (range 0-84) epg therefore the FECs had been reduced by 91% and by day 30 the FECs averaged 38.9 (range 0-195) epg therefore a FEC reduction of only 62.8%.

4.3.2.2 *Effect of treatments on the faecal nematode egg counts*

The FEC figures are all presented as back-transformed means. There were no differences in faecal nematode egg output ($P > 0.05$) between the ewes on any of the four treatments, with the exception of weeks 0, 4 and 7 where there appeared to be an interaction between MP and challenge (Figure 4.1). *Post-partum* the non-infected ewes on the HMP treatment had higher FECs than the ewes on any of the other treatments.

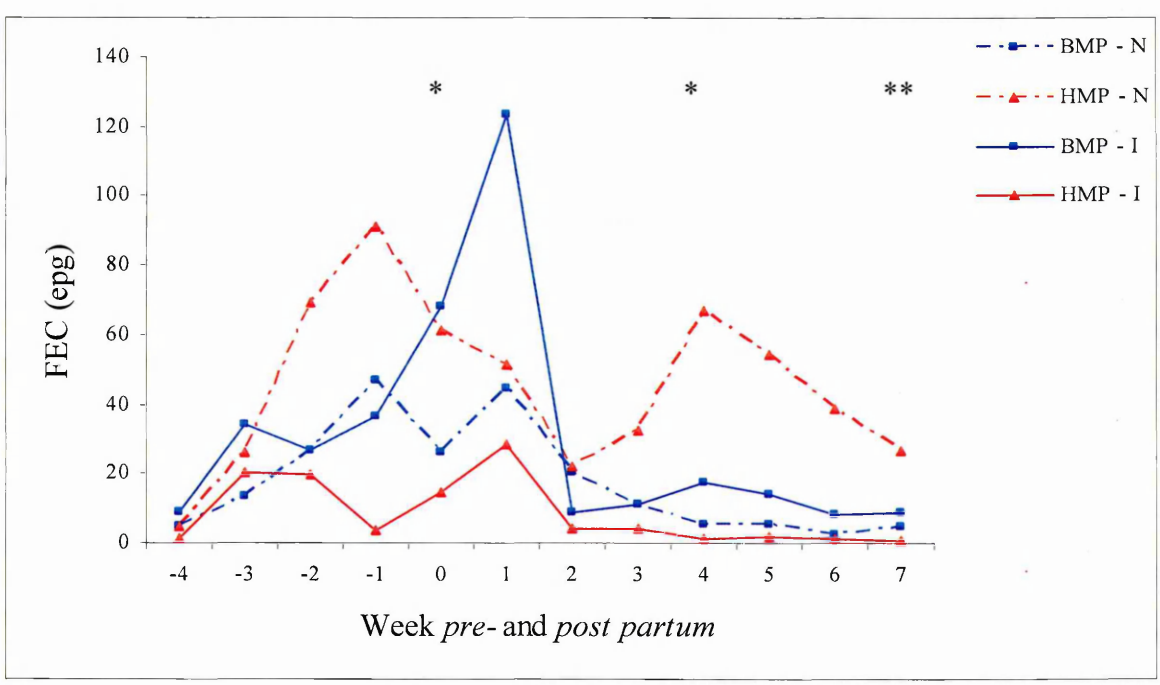


Figure 4.1. *The effect of dietary MP and nematode challenge on the ewe FECs, presented as back-transformed means. (Key, BMP = Basal Metabolisable Protein, HMP = High MP, N = not infected, I = infected. No star = non significant, * P < 0.05, ** P < 0.01.).*

4.3.2.3. *Abomasal nematode burdens*

The nematode burden figures are back-transformed means and are presented with 95% Confidence Intervals (CI) as the error (Table 4.3). There were no significant differences in ewe total nematode burden between any of the treatment groups although the high MP unchallenged group had the highest mean nematode burden.

Table 4.3. *The effect of dietary MP and nematode challenge on the total nematode burden, presented as back-transformed means with 95% confidence intervals.*

	BMP- N	HMP- N	BMP- I	HMP- I
Mean nematode burden	165	1132	768	536
Back-transformed mean (BTM)	101	1266	1293	315
Diff between BTM & lower limit 95% CI	51	786	1049	162
Diff between BTM & upper limit 95% CI	106	2079	5565	339

4.3.2.4. *Mucosal mast cell response*

The infected ewes had higher ($P < 0.001$) MMC counts than the non-infected ewes (Figure 4.2; Plate 4.1.) and dietary treatment had no effect on the MMC counts.

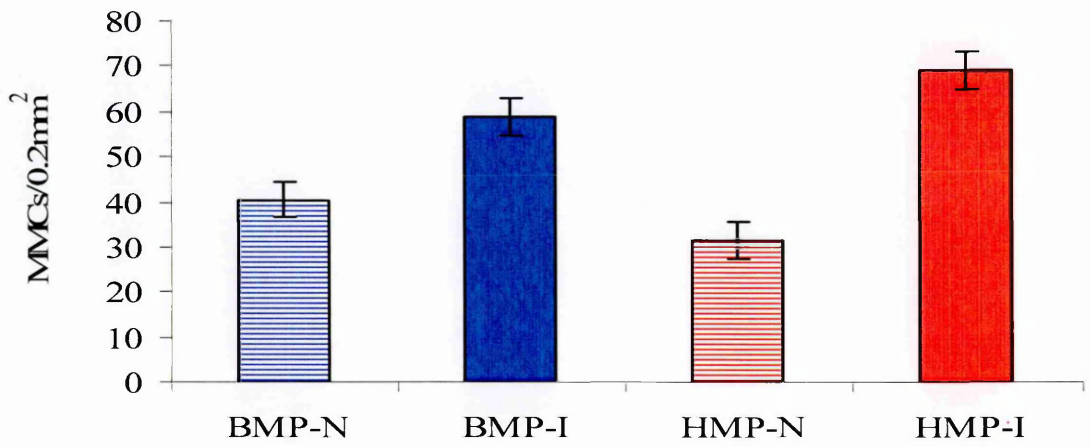


Figure 4.2. *The effect of dietary MP and nematode challenge on the ewe mucosal mast cell counts (MMCs). (Key, BMP = Basal Metabolisable Protein, HMP = High MP, N = not infected, I = infected).*

Plate 4.1. *Mucosal mast cells in the abomasal mucosa*

4.3.2.5. *Mucosal eosinophil response*

There were no significant differences in mucosal eosinophil counts amongst the treatments however the infected ewes tended to have higher numbers of mucosal eosinophils than the non-infected ewes (Figure 4.3; Plates 4.2. and 4.3.).

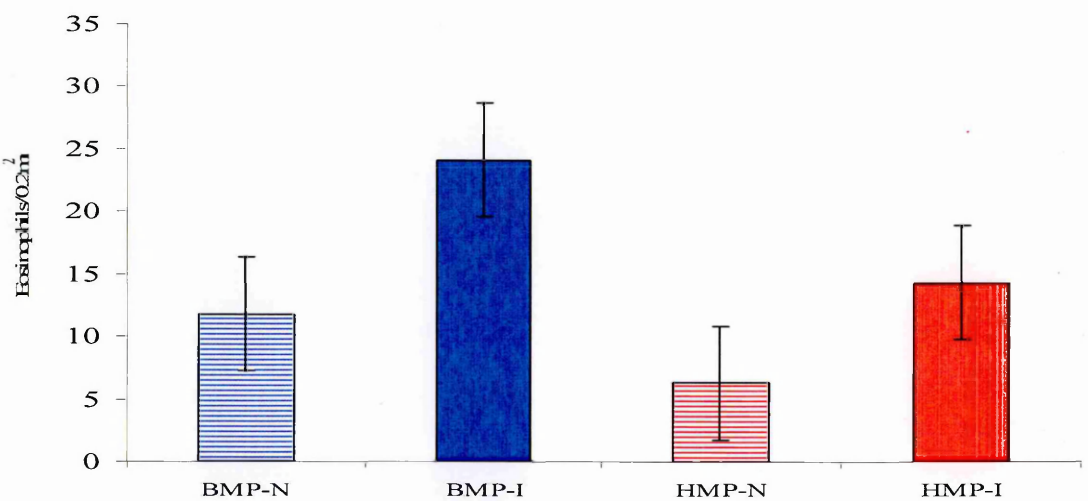


Figure 4.3. *The effect of dietary MP and nematode challenge on the ewe mucosal eosinophil counts. (Key, BMP = Basal Metabolisable Protein, HMP = High MP, N = not infected, I = infected).*

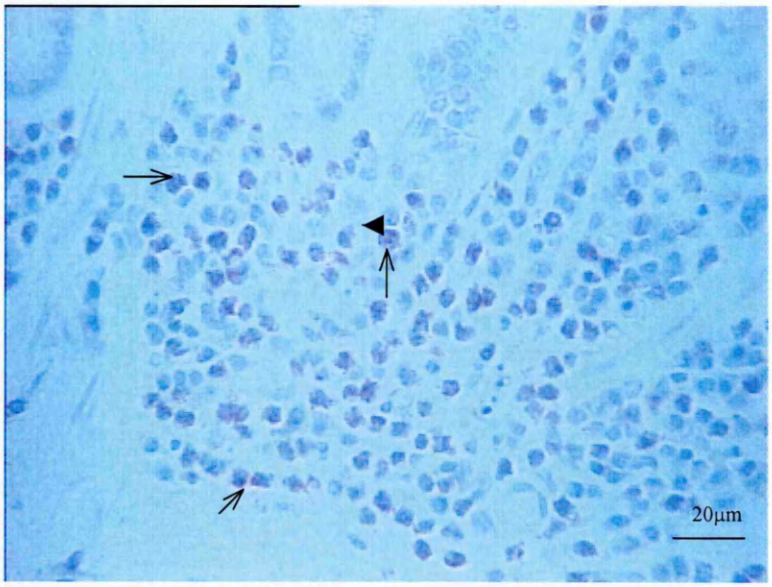
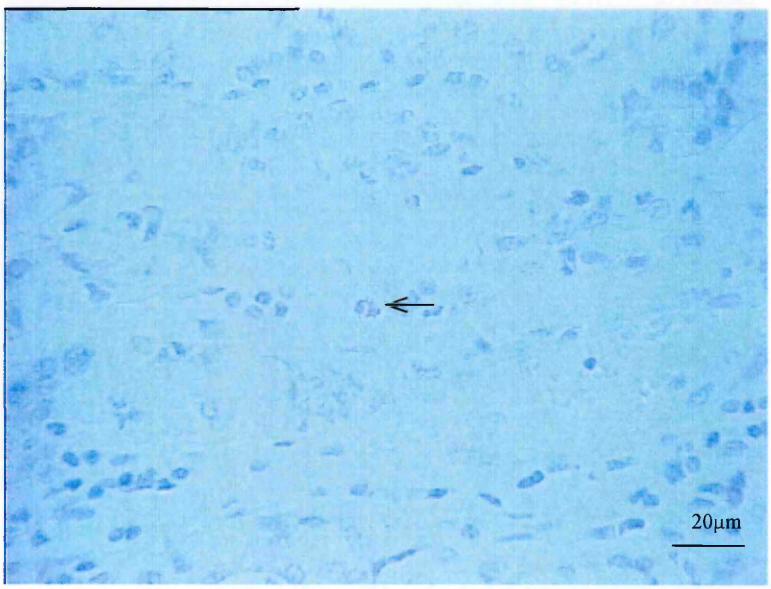


Plate 4.2. *Eosinophils in the mucosal lamina propria*



4.3. *Eosinophil in the abomasal mucosa*

4.3.3. Haematology

4.3.3.1. *Peripheral blood eosinophil counts*

The pre-challenge eosinophil concentration was recorded four weeks prior to parturition (Figure 4.4.). Challenge with larvae commenced at three weeks prior to parturition however there was no effect on eosinophil number until one week *post-partum*. From week 1 until week 5 of lactation the challenged ewes had higher ($P < 0.05$) peripheral eosinophil concentrations. In week 9 (Note: only 2 ewes group^{-1}) of lactation the Basal MP fed ewes (BMP-N and BMP-I) had lower eosinophil concentrations ($P = 0.002$) than the high MP treated ewes (HMP-N and HMP-I).

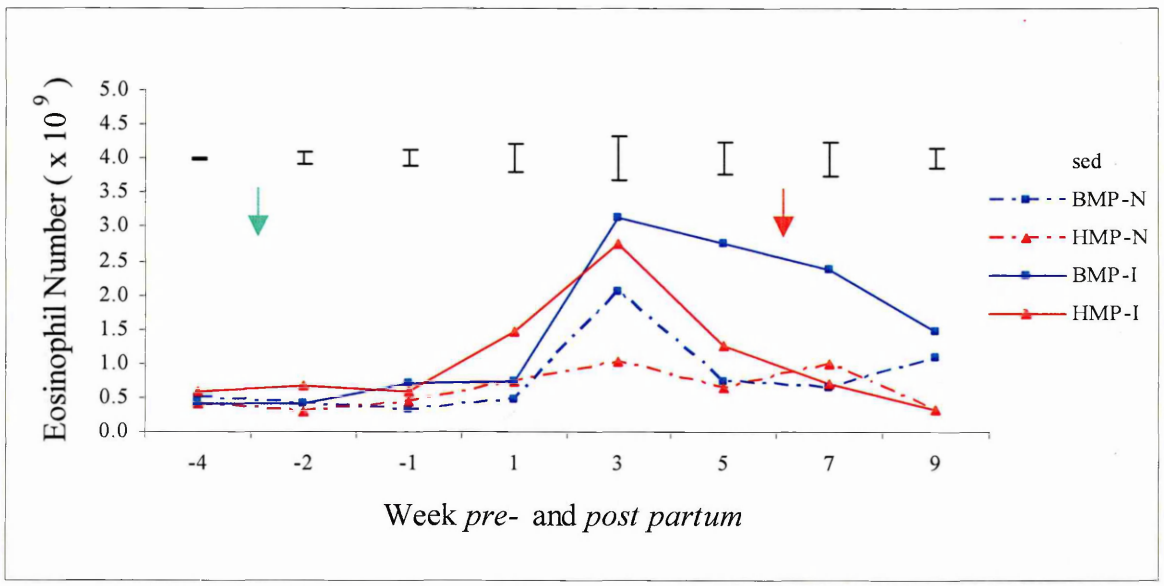


Figure 4.4. *The effect of dietary MP and nematode challenge on the ewe peripheral eosinophil concentrations*

4.3.3.2. *Haematocrit and white blood cell concentrations*

The change in haematocrit level during the *pre-partum* period was greater ($P = 0.016$) in ewes offered the high MP treatments regardless of nematode challenge (Table 4.4). None of the treatments had any effects on haematocrit levels *post-partum*.

Ewes challenged with nematode larvae had higher WBC counts in week 1 *pre-partum* ($P = 0.013$) than the non-challenged ewes (Table 4.4). Dietary treatment had no other effect on WBC counts during the *pre-partum* period. Throughout lactation the ewes challenged with

larvae had higher ($P < 0.05$) WBC counts, than the non-challenged ewes. There was an interaction between challenge and MP supply in the change in WBC count *post-partum* ($P = 0.007$). The ewes offered the high MP treatments, regardless of challenge, had a reduction in WBCs ($P < 0.001$) whereas the basal MP treated ewes had an increase in WBCs.

Table 4.4. *The effect of dietary MP and nematode challenge on ewe haematocrit and white blood cell (WBC) concentrations*

	BMP-N	HMP-N	BMP-I	HMP-I	s.e.d.	MP	INF	Int
Pre-partum haematocrit ($l\ l^{-1}$)								
Week -4	0.246	0.239	0.240	0.250	0.016	ns	ns	ns
Week -1	0.246	0.265	0.246	0.277	0.017	ns	ns	ns
Pre-partum change	+0.002	+0.026	+0.006	+0.026	0.012	*	ns	ns
Post-partum haematocrit ($l\ l^{-1}$)								
Week 1	0.273	0.276	0.264	0.290	0.019	ns	ns	ns
Week 5	0.259	0.278	0.261	0.283	0.014	ns	ns	ns
Post-partum change	-0.014	+0.02	-0.003	-0.07	0.011	ns	ns	ns
Pre-partum WBC ($10^3/mm^3$)								
Week -4	14.92	13.94	13.8	15.53	2.762	ns	ns	ns
Week -1	11.4	10.14	12.54	14.61	1.459	ns	*	ns
Pre-partum change	-3.52	-3.8	-1.26	-0.78	2.582	ns	ns	ns
Post-partum WBC ($10^3/mm^3$)								
Week 1	9.34	9.46	10.34	12.97	1.375	ns	*	ns
Week 5	9.35	8.68	12.64	11.27	1.352	ns	**	ns
Post-partum change	+0.01	-0.78	+2.3	-1.7	0.765	***	ns	**

*Key, ns = non significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$*

4.3.3.3. *Plasma pepsinogen concentrations*

Plasma pepsinogen concentrations were higher in the challenged ewes during week -1 *pre-partum* and week 1 *post-partum* compared to the un-challenged ewes (Table 4.5.). In week -1 the mean pepsinogen concentrations were 192.9mU and 80.2mU (S.E.D. 38.66, $P < 0.05$) and in week +1 the means were 320mU and 150mU (S.E.D. 54.08, $P < 0.05$) for the challenged and un-challenged ewes respectively. There were no effects of MP or interaction effects throughout the experiment.

Table 4.5. *The effect of dietary MP and nematode challenge on ewe plasma pepsinogen concentrations (mU)*

Weeks around parturition	Pepsinogen (mU)							
	BMP-	BMP+	HMP-	HMP+	S.E.D.	MP	INF	Int
(pre-challenge) -4	176	142	117	121	32.17	ns	ns	ns
-1	102	203	63	185	54.97	ns	**	ns
1	92	348	204	258	76.51	ns	**	ns
3	169	330	107	222	98.82	ns	ns	ns
5	308	224	330	212	219.1	ns	ns	ns

*Key, ns = non significant, ** $P < 0.01$*

4.3.4. Blood metabolites

4.3.4.1. *Plasma albumin concentration*

All treatment ewes were within the typical range of 24-34 g l^{-1} for sheep. Ewes offered the high MP treatment had higher plasma albumin concentrations ($P < 0.05$ *pre-partum* and $P < 0.001$ *post-partum*) than those offered the basal MP diets (Table 4.6) whilst nematode challenge had no effect.

4.3.4.2. *Plasma urea concentration*

Ewes offered the high MP treatments had higher plasma urea concentrations ($P < 0.001$) than those fed the basal MP diets (Table 4.6) with levels from the high MP treated ewes reaching a high of over 14mmol l^{-1} by the end of the experiment. Nematode challenge had no effect on plasma urea concentration ($P < 0.05$).

4.3.4.3. *Plasma total protein concentration and estimated plasma globulin concentration*

Neither protein supplementation nor nematode challenge had any effect on plasma total protein concentration *pre-partum*. In contrast, during the *post-partum* period the total protein concentration was affected by nematode challenge (Table 4.6) with ewes from the challenge treatment groups having higher total protein concentrations ($P < 0.01$) than the non-challenged ewes.

Ewes offered the high MP treatments had lower plasma globulin (total protein – albumin) concentrations ($P < 0.05$) *pre-partum* than those offered the basal MP diets. During the *post-partum* period the challenged ewes had higher ($P < 0.01$) globulin concentrations than the non-challenged ewes whilst MP supply had no effect.

4.3.4.4. *Plasma β-Hydroxybutyrate concentration*

Treatment had no significant effect on βHB concentrations *pre-partum*. In contrast, during the *post-partum* period ewes offered the high MP treatments had higher ($P < 0.01$) βHB concentrations than those offered the basal MP treatments (Table 4.6).

4.3.4.5. *Plasma glucose concentration*

During the *pre-partum* period ewes on the high MP dietary treatments had lower ($P < 0.05$) glucose levels than those on the basal MP treatments (Table 4.6). There was no effect of treatment on plasma glucose concentrations *post-partum*.

Table 4.6. *The effect of dietary MP and nematode challenge on ewe blood metabolite concentrations*

	BMP-N	HMP-N	BMP-I	HMP-I	s.e.d.	MP	INF	Int
Mean metabolites *pre-partum*								
Protein (g l^{-1})	62.5	63.2	62.2	63.5	2.04	ns	ns	ns
Albumin (g l^{-1})	27.1	29.7	26.8	28.3	1.03	*	ns	ns
Globulins (g l^{-1})	35.4	33.5	35.4	32.6	1.30	*	ns	ns
Urea (mmol l^{-1})	4.21	8.11	3.97	8.23	0.38	***	ns	ns
βHB (mmol l^{-1})	0.55	0.53	0.52	0.61	0.06	ns	ns	ns
Glucose(mmol l^{-1})	2.45	2.75	2.52	2.68	0.12	*	ns	ns
Mean metabolites *post-partum*								
Protein (g l^{-1})	66.6	66.3	68.5	70.8	1.50	ns	**	ns
Albumin (g l^{-1})	29.1	31.6	28.2	30.3	0.83	***	ns	ns
Globulins (g l^{-1})	37.6	34.8	40.3	40.5	1.98	ns	**	ns
Urea (mmol l^{-1})	5.44	14.3	5.45	15.5	0.56	***	ns	ns
βHB (mmol l^{-1})	0.59	0.63	0.53	0.69	0.04	**	ns	ns
Glucose(mmol l^{-1})	2.94	3.01	2.99	2.93	0.08	ns	ns	ns

*Key, ns = non significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$*

4.3.5. Milk yield and composition

Ewes offered the high MP treatments had higher average yields ($P < 0.01$) than the basal MP treated ewes, averaging 2321 and 1670ml day^{-1} respectively (Table 4.7). The supplementation of ewe diets with higher MP content resulted in lower average milk fat concentration ($P = 0.02$). There were no differences in the average milk protein and lactose concentrations ($P > 0.05$). In contrast, increased MP supply resulted in higher yields of fat, protein and lactose ($P < 0.001$) produced per day whilst nematode challenge resulted in lower protein ($P < 0.01$) and lactose ($P < 0.001$) yields. There was evidence of an interaction between MP supply and challenge ($P < 0.01$) on lactose production, those ewes offered the HMP diets had higher lactose yields than those not and nematode challenge resulted in a lower lactose yield when compared to the non-challenged ewes.

Table 4.7. *The effect of dietary MP and nematode challenge on ewe milk yield and composition.*

	BMP-N	HMP-N	BMP-I	HMP-I	s.e.d.	MP	INF	Int
Mean yield (ml)	1688	2406	1652	2236	306.6	**	ns	ns
Protein ($g\ kg^{-1}$)	40.2	39.6	39.2	40.4	0.78	ns	ns	ns
Protein ($g\ day^{-1}$)	67.9	95.2	64.8	90.4	1.64	***	**	ns
Fat ($g\ kg^{-1}$)	55.8	50.0	54.8	52.7	22.2	*	ns	ns
Fat ($g\ day^{-1}$)	94.2	120.4	90.5	117.7	4.33	***	ns	ns
Lactose ($g\ kg^{-1}$)	46.6	47.0	46.1	46.2	0.76	ns	ns	ns
Lactose ($g\ day^{-1}$)	78.7	113.1	76.2	103.3	1.49	***	***	**

*Key, ns = non significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$*

4.4. *Discussion*

The results presented in this chapter correspond with the findings of Experiment One in that there was no effect of dietary MP manipulation on the faecal nematode egg output of the machine-milked, dairy ewes. The results also show that challenge with larvae had no effect on FECs or worm burdens as there were no significant differences between the challenged and non-challenged ewes.

The ewes offered the High MP treatment at 1.75 x requirements (AFRC, 1993) suffered no health problems caused by high levels of protein. This was expected as Morgante (2004) noted that problems with excess dietary protein are rare in dairy ewes.

4.4.1. FECRT and anthelmintic resistance

There was evidence of a natural nematode burden during the experiment which could be indicative of an anthelmintic resistance problem (Haig *et al.*, 1995). The performance of FEC reduction tests (FECRT) prior to start of the experiment suggested that there was an anthelmintic resistance problem involving both benzimidazole and, to a lesser extent, levamisole drugs in the nematodes harboured on the pastures at Harper-Adams University College. However, the suggestion that there was a resistance to levamisole may be inaccurate as Grimshaw *et al.* (1996) suggested that the recommended dose of levamisole may not be entirely effective against levamisole-susceptible, benzimidazole-resistant immature stages of *T. circumcincta* and *H. contortus*. Due to the ewes being housed and therefore not exposed to nematodes from just prior to the benzimidazole treatment (26 days prior to the levamisole FECRT count) any larvae that were not affected by the levamisole treatment may have been in a state of hypobiosis at the time of treatment (day 19). Any conclusions based on the FECRT only are often liable to uncertainty (Cabaret and Berrag, 2004) and it may be possible that the remaining natural infection that affected this experiment may possibly have nothing to do with anthelmintic resistance. It may have been beneficial in hind-sight to have used complementary investigations such as egg hatch assays and/or genotyping for benzimidazole resistance.

4.4.2. Effect of increased MP supply and larval challenge on FECs

Due to the large difference in MP supply of 0.98 and 1.75 x requirements, it was expected that there would be a difference in FECs between the two dietary treatments as work by Coop *et al.* (1995), van Houtert *et al.* (1995), Wallace *et al.* (1999), Donaldson *et al.*

(2001) and Houdijk *et al.* (2000c; 2001c; 2003) have suggested that differences in MP supply of smaller magnitude can significantly alter the FECs of lambs and ewes. Donaldson (1998) found that differences of as little as 20g day^{-1} in estimated MP supply to periparturient ewes was sufficient to produce significant differences in FECs, considerably less than the difference used here. In the current experiment there was a predicted difference of approximately 130g day^{-1} *pre-partum* and 456g day^{-1} MP *post-partum* between the basal and high MP treatments. Although the diets provided in the current experiment were balanced for ERDP to FME ratio (10 and 11 g MJ^{-1} *pre* and *post-partum* respectively), they were not particularly scarce in MP supply at 0.99 x requirements (122 and 300 g day^{-1} *pre-* and *post-partum* respectively) and this may have been the reason for the lack of differences in FECs and worm burdens between the treatment groups.

Work by Coop *et al.* (1995), van Houtert *et al.* (1995), Wallace *et al.* (1999), Donaldson *et al.* (2001) and Houdijk *et al.* (2000c; 2001c; 2003) involved a scarcity of MP of less than 0.99 x requirements particularly during lactation, indeed Houdijk, *et al.* (2000c) offered diets calculated to provide as little as 0.85 x MP requirements (AFRC, 1993) in the last 6 weeks of pregnancy and lactation and Houdijk *et al.* (2003) offered as low as 0.65 x requirements during lactation. During pregnancy, it is difficult to provide a diet of lower than 0.97 x requirements without unbalancing the ERDP to FME ratio in the rumen (AFRC, 1993). Houdijk *et al.* (2001c) attempted to address this by offering two different diets during pregnancy; a mid-pregnancy level and a late-pregnancy level and another diet for lactation. However, in order to achieve protein concentrations of as low as 0.65 x requirements during pregnancy, the diet would be deficient in ERDP to FME (AFRC, 1993). If the ERDP to FME ratio was not balanced it would be difficult to determine if it was MP supply alone that had reduced the FECs.

4.4.3. Effect of increased MP supply and larval challenge on abomasal nematode burdens

The challenged ewes which received the Basal MP treatment had higher nematode burdens than the challenged ewes on the High MP treatments (means of 768 compared to 536 nematodes, respectively), although the difference was not significant. This was reflected in work by Houdijk *et al.* (2003) which found that an increased MP supply of 376 g day^{-1} reduced nematode burden. Despite this, the ewes receiving the HMP-N treatment had the highest burden (1132 nematodes) despite no larval challenge; this could suggest that increasing MP supply may be able to regulate larval establishment but offer no protection against established adult nematodes, although it may affect their fecundity. This contrasts with Brunsdon (1964) cited in Coop and Holmes (1996) who reported that animals with an established adult infection when transferred onto a higher protein diet had a reduction in FECs and improvement in clinical condition. By contrast, the lack of differences between the dietary treatments in pepsinogen assay results also suggests that MP may not have had an effect on larval establishment either.

4.4.4. Effect of challenge start on FECs

The onset of the PPR in FECs is not well-defined. For example, Jeffcoate *et al.* (1992) and Donaldson *et al.* (1998) noted increases in FECs from late pregnancy onwards, whereas Houdijk *et al.* (2000c) found that an elevation in FECs could commence as early as 6 weeks prior to parturition. Starting the challenge at week one of lactation may have resulted in missing an important onset of the PPR in Experiment One. Therefore even if milk yields had been high, the FECs may have remained low - certainly until week 3 of lactation due to the fact that larvae take around two to three weeks to reach maturity (Urquhart *et al.*, 1996). Even with an earlier challenge start in the current experiment at 3 weeks *pre-partum*, there was a small rise in FECs through pregnancy but again no rise at

all during lactation. This could suggest that the diets may have been responsible for the low FECs throughout the current experiment.

4.4.5. Effect of the magnitude of larval challenge on FECs and worm burden

The FECs reported here may have been as a consequence of a low infective larval dose rate of 2,000 larvae day^{-1}. Indeed, Donaldson (1998) found that a larval challenge of as much as 20,000 *T. circumcincta* larvae day^{-1} did not significantly affect the parasite status of periparturient ewes. As explained in Section 3.4.3. 2,000 larvae day^{-1} was selected as the infection rate because it was estimated to be close to the average larvae consumed at pasture. It would be of interest for future work to include a higher infection rate.

4.4.6. Effect of increased MP supply and larval challenge on ewe plasma pepsinogen levels

Pepsinogen can be used to identify an infection with *Ostertagia ostertagia* in cattle and *T. circumcincta* in sheep (Kerr, 2002). It is a gastric proteolytic enzyme present in the wall of the abomasum and is not activated to pepsin until after secretion. *O. ostertagi* has often been associated with an increase in plasma pepsinogen levels due to the mucosal damage the emerging larvae cause, which leads to pepsinogen leakage into plasma (Kerr, 2002). The greater the larval challenge, the more elevated the pepsinogen levels (Brunsdon *et al.*, 1986)

The increase in pepsinogen concentrations in plasma of the challenged ewes in week 1 *pre-partum*, two weeks after challenge commenced is in agreement with Armour *et al.* (1966), who also noticed an increase in pepsinogen at days 8-12 post-challenge with *T. circumcincta* with a peak at day 16. Armour *et al.* (1966) attributed this increase to the emergence of nematodes from the gastric glands. This theory could be supported by findings of the current work where although the un-challenged ewes were expelling nematode eggs in the faeces, there was no increase in pepsinogen levels; this suggests that

an increase in plasma pepsinogen in the challenged ewes was caused by the emergence of young adult nematodes from the gastric glands of the abomasum and not by an established adult nematode burden. The absence of significant differences in the pepsinogen assays between the ewes on the different dietary treatments suggests that increased MP has had no effect on inhibiting the establishment or emergence of the infective larvae.

4.4.7. Effect of increased MP supply and larval challenge on ewe immune parameters

4.4.7.1. *Mucosal mast cells, eosinophils and FECs*

The FECs during lactation lend credit to the theory that there was little or no nutritional scarcity to the ewes during lactation. There was a distinct reduction in FECs from week 1 of lactation through to week 2 with the exception of the ewes receiving the HMP-N treatment. The immune responses such as mast cell degranulation and eosinophil recruitment appear to be aimed at incoming larvae (Meeusen, 1999; Meeusen and Balic, 2000; Balic *et al.*, 2002). The mucosal mast cell and eosinophil counts performed in this experiment helped confirm this as the challenged ewes had significantly higher numbers of mast cells ($P < 0.001$) and eosinophils ($P = 0.026$) than the non-challenged ewes. This may explain why the reduction in FECs in the challenged groups was more pronounced than the non-challenged groups, which were carrying a nematode burden which consisted of only mature nematodes from contaminated grazing prior to group housing at 8 weeks *pre-partum*. It was likely that the challenged ewes also harboured a number of mature nematodes from the grazing as the ewes were selected from the same group of animals. It is therefore possible that the immune responses which had been mounted against the incoming larvae were responsible for reducing the fecundity of the mature nematodes as well. These results confirm, along with the pepsinogen assay results, that the infective larvae were viable.

4.4.7.2. *Peripheral blood eosinophil counts*

The lack of a difference in eosinophil counts and FECs between the challenged and non-challenged ewes during the *pre-partum* period could indicate that the ewes' immunity was not affected by pregnancy which contrasts Houdijk, *et al.* (2000c) who found that FECs can begin to increase as early as three weeks *pre-partum*. In some sensitised sheep, challenge larvae are rejected before they reach their tissue niche and this is associated with very little lymphocyte activity in the lymph nodes and no recruitment of eosinophils, referred to as rapid rejection (Balic *et al.*, 2002). However, during lactation there was an increase in peripheral WBCs, eosinophils and plasma globulins in the challenged ewes which were accompanied by a reduction in FECs suggesting that ewe immunity was more efficient during lactation than pregnancy.

The rise in peripheral eosinophils during week one of lactation was similar to the results obtained in Experiment One. The start of challenge in Experiment One was, however, in week one *post-partum,* whereas in the current experiment the challenge commenced three weeks prior to parturition. Without more studies on larval challenge during the *pre-partum* period it would be difficult to conclude that there had been an effect of pregnancy on the peripheral eosinophil counts from the current experiment and Experiment One alone.

4.4.7.3 *White blood cell counts*

The infected ewes had higher WBC counts during pregnancy and lactation ($P < 0.05$) than the un-challenged ewes. As WBCs are a collection of all the peripheral immune cells including the innate immune cells which are phagocytes and lymphocytes (which include eosinophils and mast cells), an increase in WBCs could be indicative of an infection (Roitt *et al.*, 1998). Interestingly, the ewes offered the high MP treatments had a reduction in WBC counts during lactation ($P < 0.001$) compared to an increase in WBCs in the basal MP fed ewes. Increased MP may therefore have had a detrimental effect on the immune status of the ewe which contradicts the nutrient partitioning reviews (Coop and Kyriazkis,

1999; Houdijk *et al.*, 2001a; Sykes and Coop, 2001) suggesting that MP would have a beneficial effect on ewe immunity around parturition. However, current findings are in agreement with Burkholder and Swecker (1990) who suggested that excesses of certain amino acids can have a detrimental effect on the immune system.

4.4.8. Effect of increased MP supply and larval challenge on ewe live weight and body condition score

Similar to the results in Experiment One, the ewes gained weight during lactation, except those on the HMP-I treatment. This occurred despite the consumption of diets predicted to result in a LW loss of 0.05kg day^{-1}. The ewes on any of the treatments gained condition throughout lactation with those fed the basal MP treatment gaining more condition ($P < 0.05$) than those receiving the high MP treatment. The increased LW and CS during lactation suggest a lack of nutritional scarcity across all the treatments. However, the lack of loss of weight and CS may be due to the ewes having a low mean body CS of 1.5 throughout the experiment. This would mean that mobilisation of body fat for milk production would be at a minimum as Jones and Garnsworthy (1988) suggested that cows and Wilkinson *et al.* (2000) suggested that dairy sheep with a low body condition would not mobilise body fat at the expense of the animal's health.

4.4.9. Effect of increased MP supply and larval challenge on ewe plasma metabolites

As in Experiment One ewes on the HMP dietary treatments had high plasma urea levels (8.17 and 14.4 mmol l^{-1} *pre-* and *post-partum* respectively) which were significantly higher ($P < 0.001$) than those on the LMP treatments (4.09 and 5.45 *pre-* and *post-partum* respectively). Possible reasons for this were discussed in section 3.3.6.2. The HMP treated ewes also had higher plasma albumin (discussed in section 3.3.6.2) and βHB levels (discussed in section 3.3.6.1.) ($P < 0.05$) than the LMP treated ewes, again as observed in Experiment One. During lactation, however, the challenged ewes had higher plasma

globulin levels ($P < 0.01$), which could suggest an increase in peripheral immune cells, which may have been in response to larval challenge.

4.4.10. Effect of increased MP supply and larval challenge on milk yield and composition

Yields had increased from those recorded in Experiment One. This may have been due to the increased milking frequency as this has been associated with production levels (Mills, 1989; Cant *et al.*, 2001). Ewes on the high MP treatments produced higher yields than those fed the basal MP diets at 2406 and 2236ml for the HMP-N and HMP-I treatments respectively and 1688 and 1652ml BMP-N and BMP-I treatments respectively. Yields from the HMP ewes had improved to higher than the *ad-lib,* indoor fed Friesland ewes in the study by Chikunya *et al.* (2002) where the yields averaged 2L day^{-1} and both Basal and High MP treated ewes produced higher daily yields than those from the grazed, Friesland and Finn Dorset dairy ewes used in the study by Wilkinson et al. (2000; mean 1.3L day^{-1}). The low milk yields were still lower than the estimated (<2.5L day^{-1}) which may have contributed to the restoration of immunity so soon in lactation. This may have been the reason for the low FECs during lactation despite the increased frequency of milking to 3 times a day. The other explanation would be that the development of immunity lead to a reduction in milk yield; however this would be unlikely due to there being no difference between challenged and unchallenged ewes' milk yields. The difficulty encountered with the current experiment was the apparent lack of differences in FECs between the challenged and non-challenged ewes and unexpected worm burdens present in the non-challenged ewes, results in the possibility that the mature nematode burden could have reduced the milk yield.

In agreement with Bocquier and Caja (2004), the ewes offered the HMP diets had higher milk yields ($P < 0.001$) and increased milk fat yield ($P < 0.001$) however decreased the fat percentage ($P < 0.05$). Ewes offered the HMP treatments had higher daily yields ($P <$

0.001) of protein, fat and lactose per day but parasite challenge also affected the amount of protein and lactose produced per day; challenged ewes produced less milk protein ($P < 0.01$) and lactose ($P < 0.001$) per day than the corresponding non-challenged ewes. It is hypothesised that protein was prioritised for the protection against larvae instead of milk production. By contrast, the nutrient partitioning framework described by Coop and Holmes (1996), Coop and Kyriazakis (1999) and Coop and Kyriazakis (2001), suggest that nutrients are prioritised to milk production rather at the expense of immunity.

4.5. Conclusions

Metabolisable protein had no effect on FECs of mature, periparturient, machine-milked dairy ewes, and this may have been as a consequence of low milk yields and lack of milking stimuli despite milking the ewes 3 times a day. Alternatively, the basal MP supply was not low enough to elicit a response. Further studies would benefit from a lower MP supply in the low MP treatments and a greater nutrient demand from a higher milk yield.

CHAPTER FIVE – EXPERIMENT THREE

THE EFFECTS OF METABOLISABLE PROTEIN SUPPLY AND MACHINE MILKING ON THE PERIPARTURIENT RISE IN FAECAL EGG COUNTS IN DAIRY EWES

5.1 *Introduction*

Little work has been conducted on the PPR of machine-milked dairy ewes. The results presented in Chapters three and four did not provide evidence of a beneficial effect of MP on the PPR. Previous work using ewes rearing twin lambs have suggested a beneficial effect of MP on the PPR (Houdijk *et al.*, 2000b; 2000c; 2001c; Donaldson *et al.*, 2001; Kahn, *et al.*, 2003).

In the dairy ewe, the first 4 weeks of lactation are when the greatest milk production occurs (Mills, 1989). It may be assumed that at this time the milk yield of the dairy ewe would be greater than those of the lamb-rearing ewe. However, when the lambs increase in size and milk demand the greater nutritional pressure would be on the suckling ewe. In the previous experiment, the production pressure on the dairy ewe was increased by milking 3 times a day rather than twice a day which increased milk yield slightly but had no effect on FECs. There is the possibility that suckling twin lambs could lead to a higher milk production than if machine-milked and if this were the case, then it would help explain the lack of PPR experienced in the previous two experiments. Donaldson *et al.* (1998; 2001) suggested that production pressure is a main determining factor in the development of the PPR.

The aims of the current experiment were to determine if suckling twin lambs rather than machine milking and metabolisable protein supply affected the periparturient rise in faecal nematode egg output in dairy sheep.

5.2 *Material and methods*

5.2.1 Experimental design and animals

Twenty-nine British Milk Sheep ewes (BMS) and 21 Friesland ewes were oestrus synchronised (section 2.2.1) to result in a mean parturition date of 28th April 2003. At 2 week of gestation the ewes were dosed with Noramectin™ (Ivermectin), (Norbrook labs Ltd, Newry, Co. Down, NI), at the manufacturer's recommended dose rate of 2.5ml 10kg^{-1} LW 0.8% w/v, and group-housed on straw bedding. The ewes were offered hay and grass silage *ad-lib* and remained group-housed until the experiment start at 6 weeks prior to parturition. The ewes were ultrasound-scanned to determine litter number at 77 days of gestation.

Faecal egg counts were performed before and after anthelmintic treatment at 2 weeks of gestation. Some ewes were still expelling nematode eggs and therefore all the ewes were re-dosed with the anthelmintic drug LevacurSC™ (Imidothiazole); (Levamisole hydrochloride 30mg ml^{-1} with selenium 0.32 mg ml^{-1} and cobalt 0.72 mg ml^{-1}; Intervet, Hoechst UK Ltd, Milton Keynes, U.K.), at the manufacturer's recommended dose of 7.5mg kg^{-1} LW, 11 weeks of gestation. The ewes were injected with Heptavac-P plus™, (section 3.2.1.) at 17 weeks of gestation.

Thirty-two ewes were selected for use in the experiment. The ewes ages ranged from 2 to 10 years. Twelve were scanned as single lamb bearing, 12 twin-bearing, 4 triplet-bearing and 4 quadruplet-bearing. The ewes were blocked according to breed, foetal number, live weight, condition score, previous lactation yields and allocated to 1 of 4 treatment groups (n=8, 1 quadruplet, 1 triplet, 3 twin and 3 single bearing ewes with each group containing 3 Friesland ewes and 5 BMS ewes). The experimental design was a 2 x 2 factorial design comprising of 2 dietary treatments, Low MP (LMP) and High MP (HMP) and 2 milking methods, machine-milked (M) or suckled (S). The diets differed in levels of MP supply with the low MP diet supplying 0.98 x AFRC (1993) recommended daily requirements and the high MP diet supplying 1.75 x daily requirements. There were essentially 4 diets

provided; LMP and HMP formulated to *pre-partum* requirements and LMP and HMP formulated to *post-partum* requirements (AFRC, 1993). The ewes were individually housed indoors in $3m^2$ pens and bedded on shavings with water available *ad-libitum*.

The ewes were fed the 2 *pre-partum* experimental diets (LMP an HMP) from 6 weeks prior to parturition. *Pre-partum* feeding levels were restricted (AFRC, 1993) to meet the ME requirements for litter size and weekly LW on the assumption that the ewes gained 0.025kg LW day^{-1}. Two experimental *post-partum* diets (LMP and HMP) were fed from parturition through to slaughter in week 7. These diets were fed at a restricted level according to AFRC (1993) to provide sufficient ME to produce 2.5 litres of milk day^{-1} assuming that the ewes lost 0.05kg LW day^{-1}.

Hay was offered separately in the ratio of 60:40, concentrate to hay, in both *pre-partum* and *post-partum* diets (Table 5.1). Equal amounts of ration were offered at 08.00 hours and 16.00 hours *pre-partum* and 08.00h and 15.00h *post-partum*.

Table 5.1 *Dietary ingredients offered to dairy ewes during the* pre- *and* post-partum *periods (g kg^{-1} DM)*

	Low *pre-partum*	High *pre-partum*	Low *post-partum*	High *post-partum*
Hay	400	400	400	400
Ground barley	310	205	208	104
Mol SBP	200	110	191	52
Lactamine™ *	0	183	0	302
Soya bean meal	0	12	88	50
Rape seed meal	0	0	46	0
Fat prills	31	36	21	40
Urea	19	14	14	14
Mins/vits	19	19	19	19
Molasses	18	18	18	18
Total (kg)	1000	1000	1000	1000
Predicted diet composition				
CP (g kg^{-1} DM)	134	200	171	263
ERDP 5[1]	100	96	-	-
DUP 5[1]	14	78	-	-
ERDP 8[2]	-	-	113	103
DUP 8[2]	-	-	34	128
ERDP/FME ratio	10.0	10.0	11.0	11.2
MP (g kg^{-1} DM)	77	139	106	192
MP requirement (g kg^{-1})	79	79	110	110

[1] calculated at a rumen solid phase outflow rate (r) = 0.05 hr^{-1} (g kg^{-1} DM; AFRC, 1993)
[2] calculated at a rumen solid phase outflow rate (r) = 0.08 hr^{-1} (g kg^{-1} DM; AFRC, 1993)
* Lactamine™, a metabolisable protein source of sopralin and methionine, (Trouw Nutrition, Northwich, UK.).

5.2.2 Parasite challenge

The ewes had previously been exposed to natural nematode infections prior to housing. From 6 weeks prior to parturition the ewes were dosed with approximately 4290 *Teladorsagia circumcincta* (Moredun ovine anthelmintic susceptible isolates; Moredun Research Institute, Penicuik, Scotland) infective larvae (L_3) per day until week 6 *post-partum*. This was achieved by placing 5 ml of the infective larvae in a water suspension (approximately 10000 L_3 per 5ml) on a dampened Whatman™ 1 filter paper 110mm diameter (Whatman International Ltd., Maidstone, UK), and administered as described in section 3.2.2, three times a week at 10.30 am.

5.2.3 Sample collection

Ewe live weight and condition score were determined weekly on Wednesdays at 2pm (section 2.3). Faecal samples were obtained weekly by rectal sampling, placed in a 100ml plastic container and refrigerated at 4°C. The faecal nematode egg counts (FECs) were carried out within 3 days of sampling. Sampling started from seven weeks prior to parturition, (1 week prior to experiment start). The samples were analysed for FECs using a modified floatation method (Christie and Jackson, 1982), using polyallomer centrifuge tubes to collect the nematode eggs from the meniscus (developed by Moredun Research Institute, Penicuik, Scotland) (section 2.4.2).

Blood samples were collected by jugular venepuncture into 7ml heparinised, potassium oxalate, EDTA and plain evacuated tubes (Becton, Dickinson and Company, Vacutainer Systems, Plymouth, UK), approximately one hour before the morning feed (7.30 am), fortnightly throughout the experiment. Within five hours of sampling, a small sample of whole blood was collected in vacutainers containing EDTA for eosinophil counts and haematology performed on an ABX haematological analyser (sections 2.5 to 2.5.3).

The lambs born to the ewes assigned to the machine milking treatment were removed within 48hours of parturition and the ewes commenced machine milking 3 times a day in a

Fullwood automatic goat-milking parlour at weaning. Milk samples were collected on consecutive morning, afternoon and evening milking once per week and placed in 20ml capped milk tubes (Massmould, Luton, Beds. UK) and frozen at -20°C prior to subsequent analysis. Milk yield was recorded at all 3 milkings (section 2.6). The milk yields from the ewes which were suckled were determined by back calculating from the lamb weight gain using the formula proposed by Robinson (1969); $M = 38.27W + 0.009I^2 + 411$ where M =daily milk consumption per lamb (g day^{-1}), W = body weight of the lamb (kg) and I = body weight change of the lamb (g day^{-1}). Body weight change was calculated by regression.

Samples of the treatment diets were collected weekly and frozen at -20°C prior to subsequent analysis (section 2.1).

5.2.4. Lymphocyte blastogenesis assay

Lympohocyte blastogenesis assays, using method by Mosmann (1983) were carried out on the machine-milked ewes in week 1, 3 and 6 *post-partum*. Blood was collected from the ewes at 9 am into lithium heparin vacutainers.

5.2.4.1 *Isolation of ovine peripheral blood mononuclear cells*

Blood was diluted with an equal volume of 0.15 M phosphate buffered saline (PBS), pH 7.4. A 6 ml subsample of the diluted blood was added to 6 ml of 62.5% percoll (SIGMA Chemical Co. St Louis) and centrifuged at 2000rpm for 40 minutes. The layer of white blood cells at the interface was then collected and diluted with an equal volume of 0.15 M phosphate buffered saline (PBS) at 1700 rpm for 7 minutes. The resultant packed cells were then washed twice by repeated resuspension and centrifugation with tissue culture medium (TCM), made up of 25 ml neonatal calf serum, 13.5 ml sodium bicarbonate (SIGMA-Aldrich Co. Ltd, Irvine, UK), 5 ml L-Glutamine, 1 ml Amphoteracin B (SIGMA-

Aldrich Chemie, Gmbh, Germany), 5 ml Getamycin Sulphate, 500 ml of RPMI 1640 (SIGMA Aldrich Co. Ltd, Irvine, UK). Packed cells were resuspended in 2ml of TCM.

5.2.4.2 *Counting viable cells*

A 100 µl sub-sample was added to 100 µl of 0.2% Nigrosine solution in a 1.5 ml micro-centrifuge tube for staining. Viable mononuclear cells were then counted using a haemocytometer (Weber Scientific International Ltd, Middlesex, UK) under a 40 x lens.

5.2.4.3. *Cell culture*

Cells were diluted to a concentration of 2 x 10^6 ml^{-1} with TCM and 180 µl of cell suspension was pipetted into triplicate wells on a sterile 96-well microtest™ U-bottom tissue culture plate (Becton Dickinson Labware, France).

Twenty microlitres of 5µg ml^{-1} Concanavalin A (Con A; Aldrich Chemical Company Inc. USA), 20µL of 5µg ml^{-1} Lectin Phytolacca americana (Pokeweed Mitogen; PWM) were then added to the triplicate wells and 20 µl RPMI 1640 was added to the control wells. The plates were placed in an incubator (SANYO CO_2 Incubator, model: MCO – 15AC) at 37°C in 5% CO_2. After 48 hours, 20 µl of 5mg ml^{-1} of 3-(4-5-dimethyldiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in PBS was added and the plates incubated for a further 4 hours.

The plates were then centrifuged at 1400 x g for 5 minutes (2000 series, Centurion). The supernatent was aspirated off using a multi-channel pipette. Two hundred microlitres of dimethyl sulphoxide (DMSO) (BDH laboratory supplies, Poole, UK) working solution (180ml DMSO to 20 ml 1M HCl) was then added to the wells. The plates were left for fifteen minutes before reading on an ELISA reader (Microplate Manager, Bio-Rad Laboratories) at 570 nm wavelength with a reference wavelength of 630nm.

5.2.5. Statistical analysis

Data was analysed for a normal distribution and skewed data transformed by Log_{10} (n+1). Statistical analysis was performed using Genstat release 7.2. Lawes Agricultural Trust (2004) and analysed as a 2 x 2 factorial analysis of variance (ANOVA), using the factors protein level (MP), milked or suckled (S) and their interaction (Int). The skewed data is presented as the back transformed means (BTM) with the difference between the BTM and the back transformed 95% confidence interval (CI) as the error bars.

5.3 *Results*

5.3.1. General sheep health

Throughout the experiment, there were no clinical signs (section 3.3.1) of infection with *T. circumcincta.* Five of the ewes had foot rot from the onset of the trial and were treated (section 2.3.). Two ewes had high FECs pre-challenge at week -7 (one ewe on the HMP-S and one on the HMP-M treatment), and these ewes were omitted from the statistical analysis. One ewe on the HMP-M treatment was omitted from the lactation results as she had mastitis from parturition and produced no milk. This resulted in six ewes on the HMP-M treatment, seven on the HMP-S treatment and eight on the LMP-M and LMP-S treatments.

5.3.1.1. *Ewe live weight, condition score and lamb birth weight*

At week six *pre-partum,* ewe live weight was similar amongst treatments and averaged 64.9kg. During the *pre-partum* period the ewes on the HMP diets gained more LW ($P < 0.001$) than those on the LMP dietary treatments (Table 5.2.). The HMP ewes remained at a higher LW throughout lactation ($P < 0.05$) than the ewes on the LMP treatment. Suckled ewes lost, on average, 3.18kg LW during lactation whereas the milked ewes gained on average, 1.79kg LW throughout lactation ($P < 0.001$). There were no differences in CS amongst the treatments throughout the experimental period although in week 1 *pre-partum,*

the ewes fed the HMP treatments tended to have a higher body CS (P = 0.09) than the LMP ewes. There were no differences in lamb birth weights amongst the treatments. although the suckled lambs on the ewes offered the HMP diets had a slightly higher daily weight gain (P = 0.06) than the lambs on ewes receiving the LMP diet.

Table 5.2. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on ewe live weight, condition score and lamb birth weight.*

	LMP-M	LMP-S	HMP-M	HMP-S	s.e.d.	MP	S	Int
Pre-partum LW (kg)								
Week -6	64.8	62.5	66.5	65.7	4.51	ns	ns	ns
Week -1	70.8	68.5	80.0	80.4	5.27	**	ns	ns
Pre-part. change	+5.95	+6.02	+13.5	+11.8	1.689	***	ns	ns
Pre-partum CS								
Week -6	2.5	2.5	2.3	2.8	0.260	ns	ns	ns
Week -1	2.4	2.3	2.5	2.6	0.245	ns	ns	ns
Pre-part. change	-0.13	-0.22	+0.13	-0.25	0.188	ns	ns	ns
Post-partum LW (kg)								
12 hr *post-partum*	61.8	61.7	69.2	72.6	5.07	*	ns	ns
Wk 6 *post-partum*	63.9	59.2	68.1	66.9	3.94	*	ns	ns
Post part. change	+2.03	-2.23	+1.54	-4.13	1.835	ns	***	ns
Post-partum CS								
12 hr *post-partum*	2.4	2.3	2.5	2.6	0.245	ns	ns	ns
Wk 4 *post-partum*	2.1	2.0	2.2	2.3	0.179	ns	ns	ns
Post part. change	-0.31	-0.22	-0.29	-0.25	0.160	ns	ns	ns
Lamb weights								
Mean bwgt (kg)	3.39		3.57		0.42	ns	n/a	n/a
Mean wt gain (g day^{-1})	-	202	-	256	27.5	ns	n/a	n/a

*Key, ns = non significant, n/a = not applicable, * P < 0.05, ** P < 0.01, *** P <0.001*

5.3.2 Parasitology

5.3.2.1 *Faecal nematode egg counts*

At six weeks *pre-partum,* all the FECs were similar amongst treatments and averaged, 1.4 epg. From four weeks *pre-partum* through to week 1 of lactation, ewes offered the HMP diets had lower FECs (P < 0.05) than those fed the LMP diets (Figure 5.1.). During

lactation, ewes suckling lambs had higher FECs ($P < 0.001$) from week 3 of lactation through to week 7. The ewes on the HMP diets had lower FECs from week 3 of lactation ($P < 0.01$) and there was a significant interaction between milking treatment and dietary treatment in weeks 3, 4 and 7 of lactation ($P < 0.05$); the suckled ewes offered the LMP diet had significantly higher FECs than the ewes from the other 3 experimental groups.

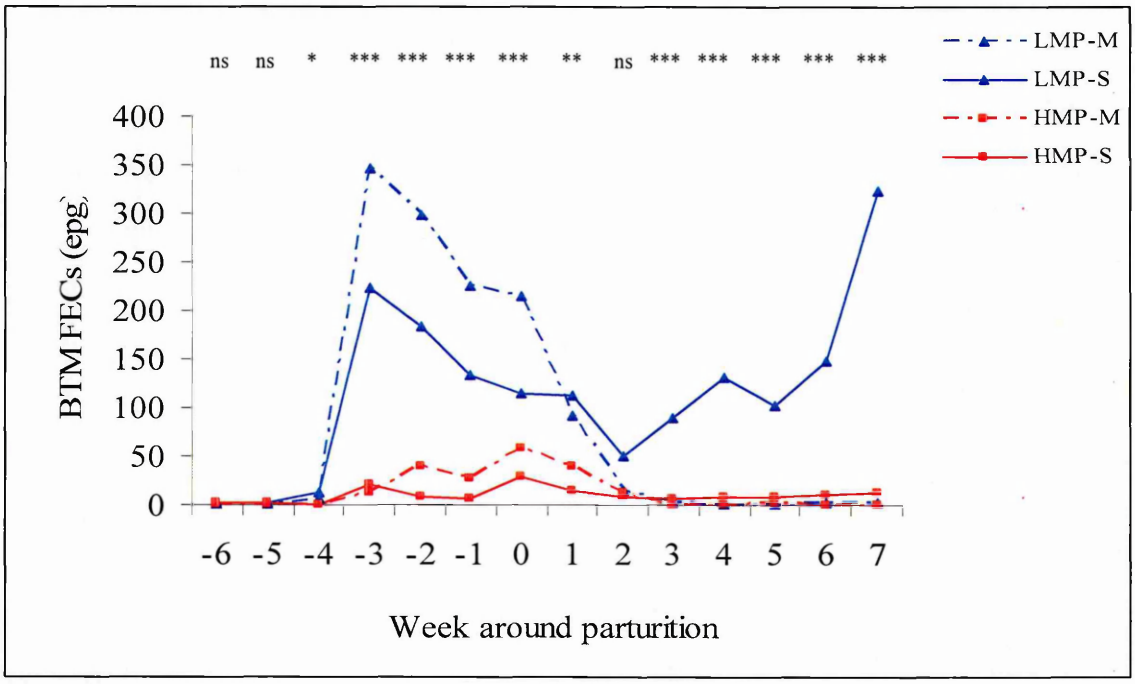


Figure 5.1. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on ewe FECs (presented as back-transformed means from $log_{10}(n+1)$ transformed data with F-probabilities as stars. Key, ns = non significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).*

5.3.2.2. *Abomasal nematode burdens*

The ewes which suckled lambs (S) had higher nematode burdens than the machine-milked ewes (M), averaging 12406 and 2396 nematodes, respectively ($P = 0.004$) (Figure 5.2.). There was no effect of dietary MP on the nematode burdens ($P = 0.515$) and no interaction between the milking and dietary treatment ($P = 0.995$).

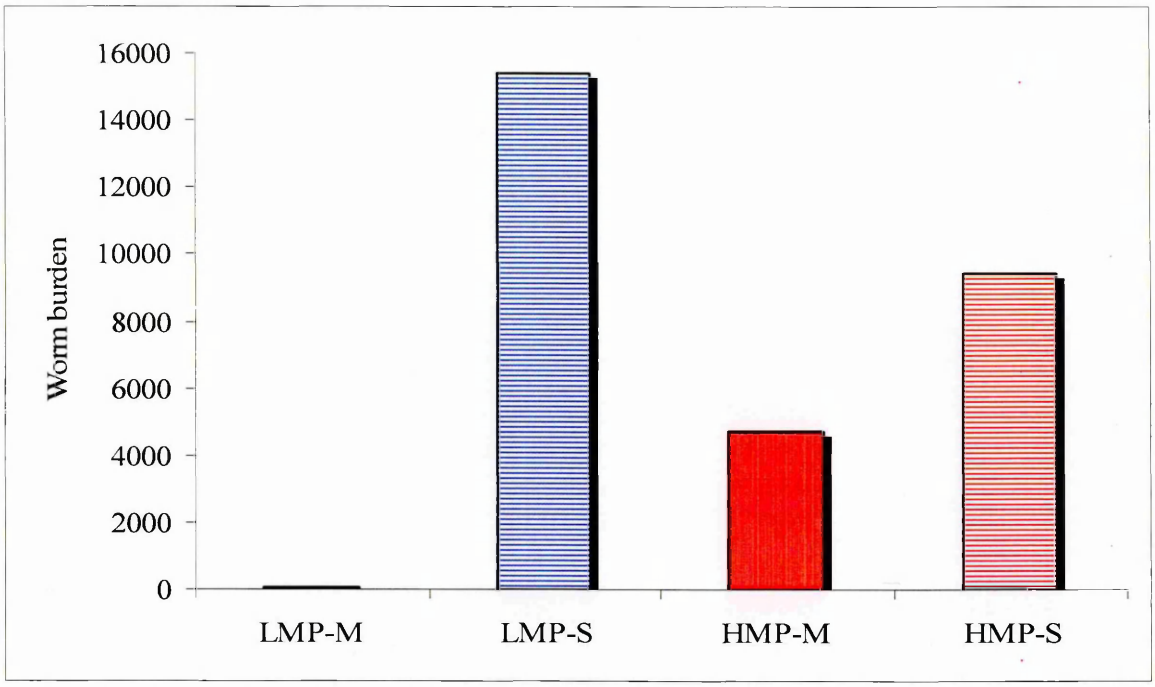


Figure 5.2. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on ewe worm burdens (presented as back-transformed means from $log_{10}(n+1)$ transformed data).*

5.3.2.3. *Nematode fecundity results*

The ewes which suckled (S) had higher nematode eggs *in utero* than the machine-milked ewes (M) ($P < 0.004$) (Figure 5.3.). There were no effects of MP supply ($P = 0.746$) on the *in utero* egg counts and no interactions ($P = 0.594$).

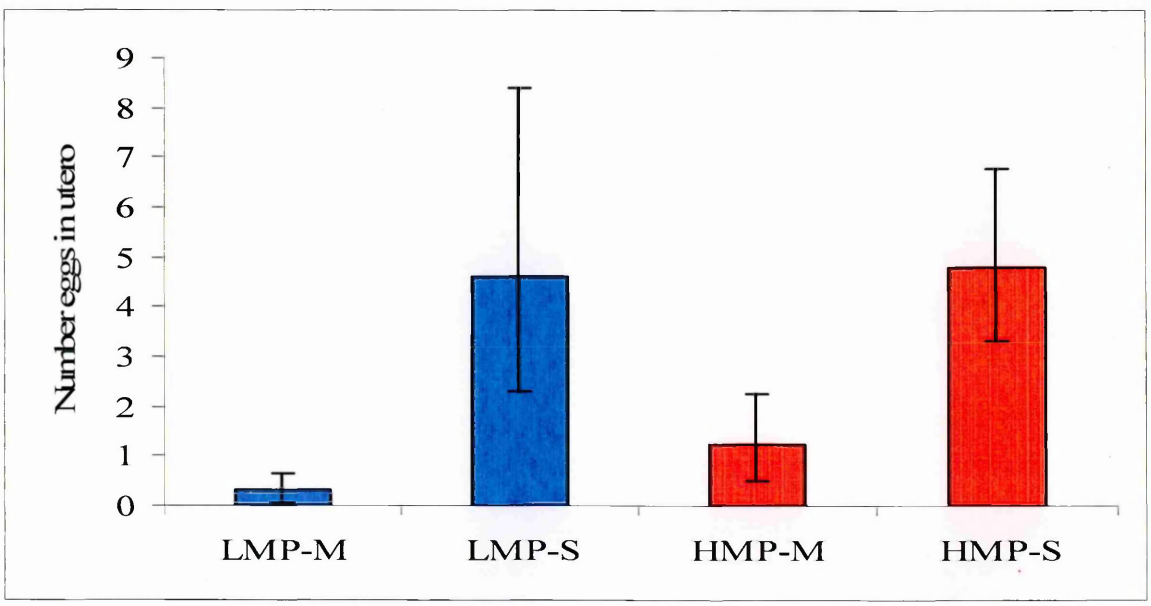


Figure 5.3. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on the* in utero *nematode egg counts. (Results are presented as back-transformed means with 95% CI as bars).*

5.3.2.4. *Mucosal mast cell response*

There were no significant differences in MMCs between the treatments (Figure 5.4.). There was a tendency towards an interaction between the dietary treatments and the milking treatments (P = 0.05), the twin-suckled ewes fed the HMP diet and the milked-ewes offered the LMP diet had lower MMCs than the LMP-S and HMP-M treatments.

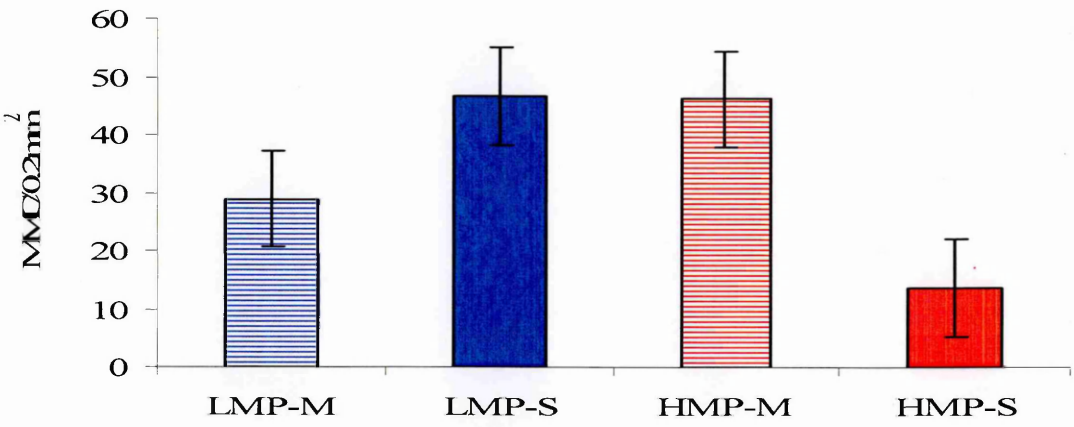


Figure 5.4. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on the ewe mucosal mast cell (MMC) counts per 0.2 mm^2 of abomasal mucosa*

5.3.2.5. *Mucosal eosinophil response*

There was no effect of MP supply or milking method on the ewe mucosal eosinophil counts (P > 0.05); (Figure 5.5.).

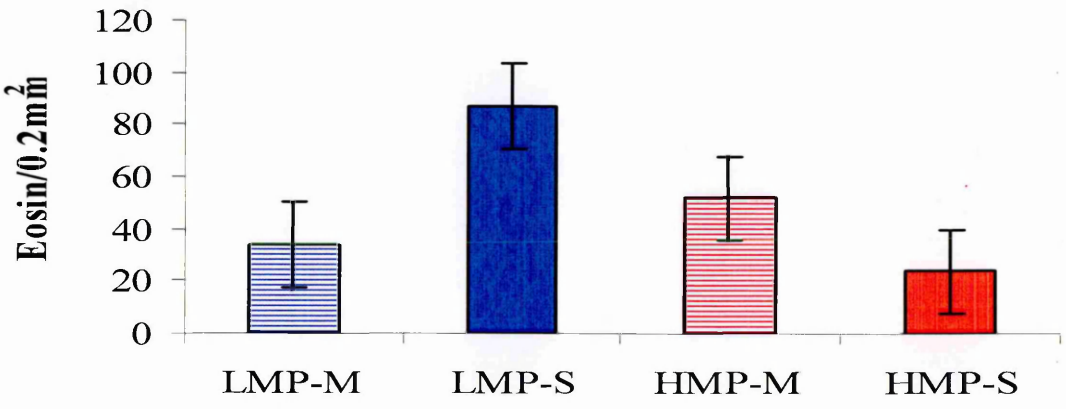


Figure 5.5. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on the ewe eosinophil counts per 0.2 mm^2 of abomasal mucosa*

5.3.3. Haematology

5.3.3.1. *Peripheral blood eosinophil counts*

The counts performed in week -5 demonstrate the number of peripheral eosinophils after one week of challenge. The ewes assigned to be machine-milked (M) had significantly higher (P = 0.045) eosinophil counts than the ewes assigned to the suckling treatment (S) in week -5. During weeks -3 and -1, there were no differences in peripheral eosinophil counts (P > 0.05) (Figure 5.6.). However, from week 3 of lactation, the suckled ewes had lower eosinophil counts (P < 0.05) than the machine-milked ewes.

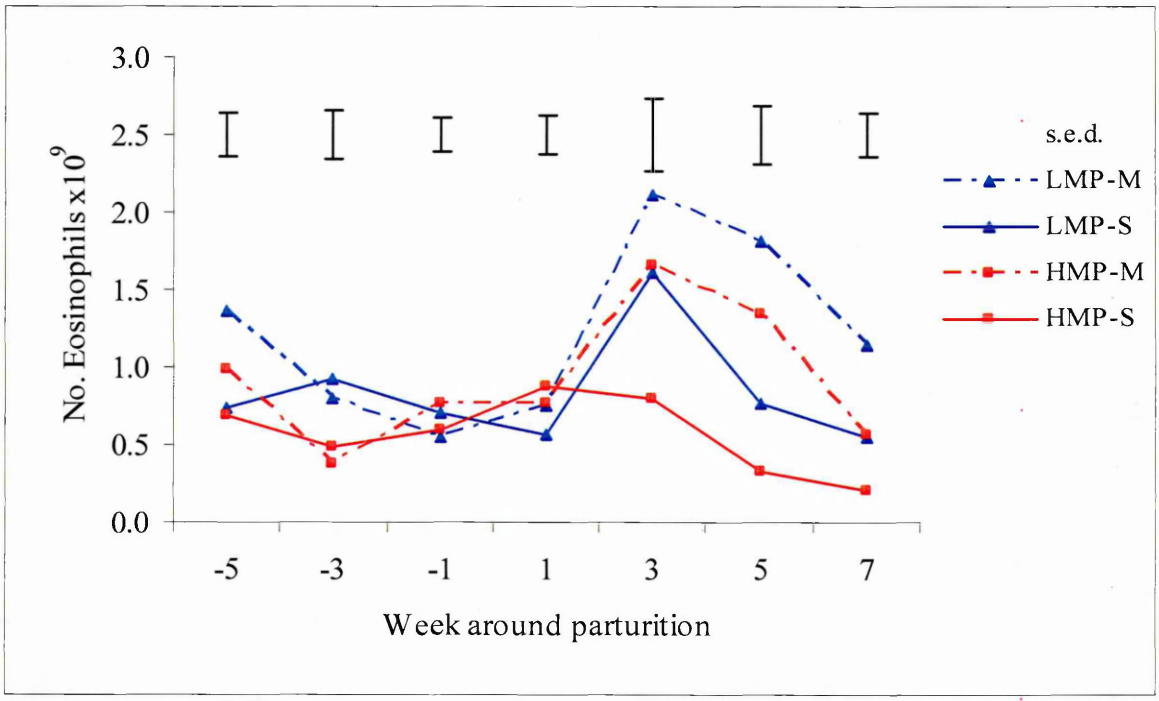


Figure 5.6. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on ewe peripheral eosinophil concentration (x10^9 ml^{-1}).*

5.3.3.2. *Haematocrit and white blood cell concentrations*

Blood haematocrit levels at seven weeks *pre-partum* were similar amongst treatments, averaging 0.29 l l^{-1}. During week 1 *pre-partum,* ewes offered the HMP treatment had higher haematocrit levels (P = 0.014); (Table 5.3.). The haematocrit levels of the ewes offered the LMP diet reduced whilst the ewes offered the HMP diet had an increase (P = 0.002) *pre-partum*. During lactation, the average haematocrit level was higher for the ewes

on the HMP treatments than the ewes on the LMP treatments ($P < 0.01$), and there was no effect of suckling or machine milking.

Mean white blood cell counts were 12.6 and 11.7 x 10^3/mm^3, *pre-* and *post-partum* respectively, there were no significant differences in WBC concentrations at any time throughout the experimental period (Table 5.3.).

Table 5.3. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on ewe haematocrit and white blood cell (WBC) concentrations*

	LMP-M	LMP-S	HMP-M	HMP-S	s.e.d.	MP	S	Int
Pre-partum haematocrit (l l^{-1})								
Week -7	0.291		0.282		0.013	ns	n/a	n/a
Week -1	0.274		0.313		0.015	*	n/a	n/a
Pre-partum change	-0.017		+0.027		0.013	**	n/a	n/a
Post-partum haematocrit (g kg^{-1})								
Week 1	0.266	0.242	0.294	0.289	0.014	**	ns	ns
Week 7	0.260	0.248	0.281	0.298	0.016	**	ns	ns
Post-partum change	-0.06	+0.06	-0.13	+0.09	0.014	ns	ns	ns
Pre-partum WBC (10^3/mm^3)								
Week -7	13.3		12.6		1.31	ns	n/a	n/a
Week -1	12.4		12.2		0.90	ns	n/a	n/a
Pre-partum change	-0.94		-0.33		1.15	ns	n/a	n/a
Post-partum WBC (10^3/mm^3)								
Week 1	12.5	11.8	10.8	12.8	1.97	ns	ns	ns
Week 7	11.6	13.0	11.0	10.3	1.97	ns	ns	ns
Post-partum change	-0.85	+1.14	+0.27	-2.50	1.96	ns	ns	ns

*Key, ns = non significant, n/a not applicable, * $P < 0.05$, ** $P < 0.01$.*

5.3.3.3. *Lymphocyte blastogenesis performed on blood from the milked ewes*

The level of metabolisable protein in the diet had no effect on the lymphocyte responses to Concanavalin A (ConA) mitogen (P = 0.430) or to Lectin Phytolacca americana (Pokeweed) (PWM) mitogen (P = 0.430) or to the control (RPMI 1640) when analysed by repeated measures (Genstat release 7.2. Lawes Agricultural Trust, 2004) (Table 5.6.). For the ewes on both treatments, time had an effect on the lymphocyte response to ConA (P =

0.036), PWM (P = 0.021) and the control medium (P = 0.046). For the two mitogens (ConA and PWM) and the controls, the optical densities (OD_{570}) increased with time regardless of dietary treatment. There were no significant time x level of dietary MP interactions on the lymphocyte responses to ConA, PWM and the control medium.

Table 5.4. *The effect of dietary low (L) or high (H) metabolisable protein (MP) on lymphocyte blastogenesis response to Concanavalin A (Con A), Lectin Phytolacca Americana (PWM) and control (RPMI 1640) mitogen in machine-milked ewes infected with* Teladorsagia circumcincta.

Time (Week of lactation)	Mitogen	OD_{570} High MP	OD_{570} Low MP	S.E.D.	*P*
1	ConA	0.136	0.121	0.0225	ns
3	ConA	0.170	0.134	0.0508	ns
6	ConA	0.227	0.260	0.0728	ns
1	PWM	0.105	0.098	0.0197	ns
3	PWM	0.141	0.072	0.0450	ns
6	PWM	0.253	0.195	0.0869	ns
1	Control	0.064	0.068	0.0125	ns
3	Control	0.077	0.076	0.0355	ns
6	Control	0.207	0.120	0.0715	ns

5.3.4. Blood metabolites

5.3.4.1. *Plasma albumin concentration*

The ewes offered the HMP treatment had higher albumin concentrations (P < 0.001) than the ewes offered the LMP diets both *pre-partum* (mean values of 26.3 and 30g l^{-1} LMP and HMP treatments respectively) and *post-partum* (mean values of 28.3 and 32.6g l^{-1} LMP and HMP treatments respectively); (Table 5.4.). Milking method had no effect on plasma albumin levels *post-partum* (P = 0.335).

5.3.4.2. *Plasma urea concentration*

The ewes offered the HMP diet had higher urea concentrations (P < 0.001) than the ewes offered the LMP diet both *pre-partum* (mean values of 4.8 and 8.0 mmol l^{-1} LMP and HMP treatments respectively) and *post-partum* (mean values of 6.8 and 15.8 mmol l^{-1}

LMP and HMP treatments respectively); (Table 5.4.). Milking method had no effect on plasma urea levels *post-partum* ($P = 0.495$).

5.3.4.3. *Plasma total protein concentration and estimated plasma globulin concentration (total protein – albumin)*

There were no significant differences in total protein concentration or globulin concentration amongst treatments *pre-partum* (Table 5.4.). *Post-partum,* ewes offered the HMP treatment had higher total protein concentrations ($P = 0.01$) than the ewes offered the LMP treatments whilst ewes suckling lambs had higher levels than machine-milked ewes ($P = 0.037$). There were no significant differences in globulin concentrations *post-partum.*

5.3.4.4. *Plasma β-Hydroxybutyrate concentration*

Dietary treatment had no effect on plasma βHB concentration *pre-partum.* In contrast, during the *post-partum* period, the ewes offered the HMP diets had higher ($P = 0.049$) βHB concentrations than the ewes offered the LMP diets with mean values of 0.46 and 0.55mmol l^{-1} for the LMP and HMP treatments, respectively (Table 5.4). The ewes suckling lambs also had higher βHB levels than the machine-milked ewes ($P = 0.034$) with mean values of 0.44 and 0.55 mmol l^{-1} for the milked and suckled ewes, respectively.

5.3.4.5. *Plasma glucose concentration*

There were no effects of metabolisable protein supply or milking on plasma glucose levels throughout the experimental period (Table 5.5.).

Table 5.5. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on the mean* pre- *and* post-partum *ewe blood metabolites.*

	LMP-M	LMP-S	HMP-M	HMP-S	s.e.d.	MP	S	Int
Mean metabolites *pre-partum*								
Protein (g l^{-1})	64.0		66.6		1.63	ns	n/a	n/a
Albumin (g l^{-1})	26.3		30.0		0.75	***	n/a	n/a
Globulins (g l^{-1})	37.7		37.0		1.84	ns	n/a	n/a
Urea (mmol l^{-1})	4.77		8.04		0.40	***	n/a	n/a
βHB (mmol l^{-1})	0.46		0.47		0.07	ns	n/a	n/a
Glucose (mmol l^{-1})	2.62		2.80		0.09	ns	n/a	n/a
Mean metabolites *post-partum*								
Protein (g l^{-1})	68.8	70.7	71.5	76.0	2.05	*	*	ns
Albumin (g l^{-1})	28.4	28.2	31.7	33.5	1.15	***	ns	ns
Globulins (g l^{-1})	40.4	42.5	39.8	42.6	2.31	ns	ns	ns
Urea (mmol l^{-1})	6.67	6.98	15.5	16.0	0.76	***	ns	ns
βHB (mmol l^{-1})	0.40	0.49	0.48	0.61	0.07	*	*	ns
Glucose (mmol l^{-1})	2.94	2.90	2.78	2.84	0.13	ns	ns	ns

*Key, ns = non significant, n/a not applicable, * $P < 0.05$, *** $P < 0.001$*

5.3.5. Ewe milk yields and the composition of milk produced by the machine-milked ewes

The ewes which suckled lambs had higher milk yields ($P = 0.001$) than the machine-milked ewes (Table 5.5.). There were no differences in milk fat or lactose concentrations or yields although the ewes on the LMP diet had higher ($P < 0.05$) protein concentrations and yield than the ewes offered the HMP diet.

Table 5.6. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on ewe milk yield and composition.*

	LMP-M	LMP-S	HMP-M	HMP-S	s.e.d.	MP	S	Int
Mean yield (ml)	1599	1957	1610	2496	243.5	ns	**	ns
Yield change (ml)	-379	+460	-421	+569	186.2	ns	***	ns
Protein (g kg^{-1})	39.9	-	37.2	-	0.098	*	n/a	n/a
Protein (g day^{-1})	63.8	-	59.9	-	0.157	*	n/a	n/a
Fat (g kg^{-1})	53.6	-	53.9	-	0.173	ns	n/a	n/a
Fat (g day^{-1})	85.8	-	86.7	-	0.279	ns	n/a	n/a
Lactose (g kg^{-1})	45.2	-	46.8	-	0.085	ns	n/a	n/a
Lactose (g day^{-1})	72.4	-	74.8	-	0.136	ns	n/a	n/a

*Key, ns = non significant, n/a = not applicable, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$*

The machine-milked ewes' daily yield had decreased by week 6 of lactation whereas the ewes suckling lambs produced higher daily yields in week 6 than in week 1 of lactation ($P < 0.001$) (Figure 5.7).

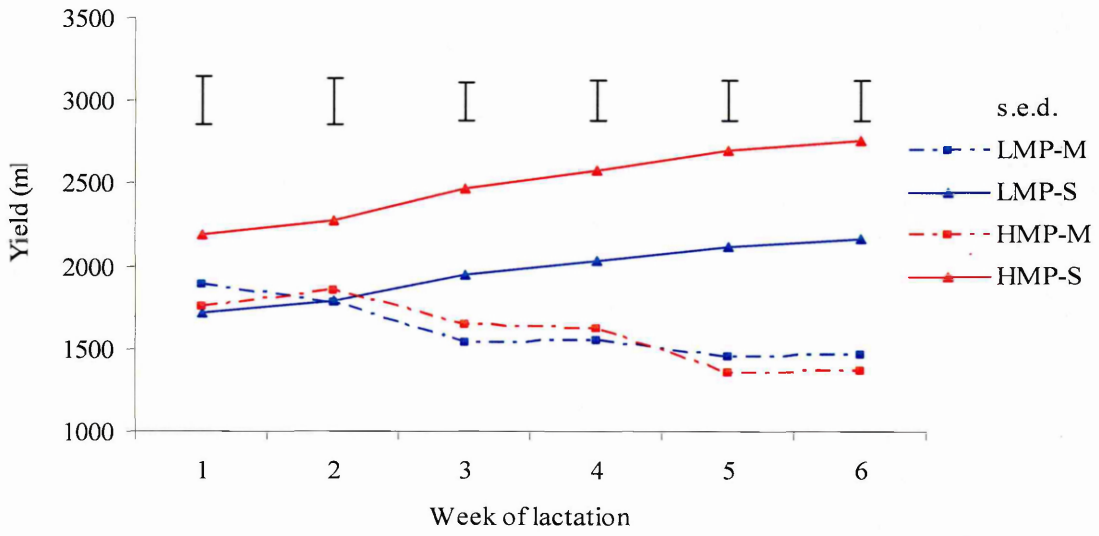


Figure 5.7. *The effect of dietary low (L) or high (H) metabolisable protein (MP) and suckling (S) or machine milking (M) on ewe milk yield.*

5.4 Discussion

The results of the current experiment complement an increasing body of evidence (e.g. Donaldson *et al.*, 2001; Houdijk *et al.*, 2001a; 2003) that increasing the supply of dietary MP can reduce the faecal nematode egg output of periparturient ewes and consequently reduce the dependency on chemoprophylaxis. The study also revealed that machine-milked dairy ewes did not have as great a rise in FECs *post-partum* as ewes which suckled twin lambs.

5.4.1. Effect of treatment on FECs and worm burdens

Two weeks after nematode challenge and the consumption of the dietary treatments commenced (4 weeks *pre-partum*), ewes offered the HMP diets had lower FECs than those

offered the LMP diets ($P < 0.05$), and through the rest of pregnancy and the first week of lactation this was highly significant. This observation correlates with the findings of Houdijk *et al.* (2000b; 2000c; 2001c), Donaldson *et al.* (2001), Kahn *et al.* (2003) that increased MP supply can reduce the faecal egg output of the periparturient ewe. However, by week 2 of lactation there were no differences between the FECs of the machine-milked ewes on the different dietary treatments. At week 3 of lactation through until slaughter the milking method was the main effect on the FECs whereby the ewes suckling twin lambs had higher FECs ($P < 0.001$) than the machine-milked ewes, High dietary MP supply still lowered the FECs of the suckled ewes ($P < 0.01$) and there was evidence of an interaction between milking and dietary treatments in weeks 3, 4 and 7. In those weeks, the ewes on the LMP dietary treatment which were suckling twin lambs had higher FECs than any of the other ewes ($P < 0.05$).

These results help to explain the findings of the previous two experiments whereby when lactation commenced, the machine-milked dairy ewes, regardless of dietary treatment, cease to be affected by the PPR in FEC. The FECs decreased dramatically at the commencement of machine-milking and this occurred in parallel with the increases in LW during lactation and the lower than anticipated milk yields. Also, there were significantly more nematodes in the abomasa of the twin-suckling ewes than the machine-milked ewes regardless of dietary MP supply ($P = 0.004$).

Whether the low yields arose from the ewes defending against the parasites or whether the yields were low because of an external factor such as the rupture of the maternal bond, an insufficient milking frequency or the dietary treatments, and as a consequence the ewes were successful in defending against the parasites is unclear. It is likely that the ewes may focus nutrient demands towards reproduction and the rearing of offspring rather more efficiently with the stimulation from the actual lamb. Perhaps with machine milking alone there was no incentive to produce milk at the expense of the ewe's own well-being.

In Experiment Two, no differences in the milk yield were obtained between challenged and non-challenged ewes. Had there been a negligible worm burden in the non-challenged ewes and no change in the milk yield between the challenged and non-challenged ewes then this may have demonstrated that the milk yields were lower due to the actual act of machine milking rather than an affect from the worm burden/challenge. However, as this was not the case and since Leyva *et al.* (1982) suggests that *T. circumcincta* can significantly reduce milk yields it may be assumed that a trickle infection of 4,300 larvae day^{-1} had some influence on reducing the milk yields in the machine-milked ewes if not all the ewes .

Although the ewes on the HMP treatment that suckled lambs had slightly higher FECs than the machine-milked ewes they were still significantly lower than the ewes which were offered the LMP treatment demonstrating that increased MP does decrease FECs of periparturient ewes.

5.4.2. Effect of treatments on ewe haematological parameters and immune function

During the *post-partum* period from week three until week seven ewes suckling twin lambs had lower peripheral eosinophils than the machine-milked ewes ($P < 0.05$). Eosinophils are involved in the defence against gastrointestinal nematodes as described in the previous two chapters and often there is a noticeable rise in peripheral and tissue eosinophils in response to invading larvae in previously exposed animals (Balic *et al.*, 2000a).

Dawkins *et al.* (1989) and Buddle *et al.* (1992) suggested that eosinophilia is more likely to be a measure of the host's resistance to gastrointestinal nematodes, rather than an indication of nematode burden. The results of the current experiment suggest a slight negative correlation ($r(17) = -0.402$, $P<0.05$) between eosinophil number and nematode burden, therefore a higher eosinophil concentration resulted in a lower nematode burden, similar to the findings of van Houtert *et al.* (1995) who also found that worm expulsion increased with increasing levels of fish meal supplementation and that expulsion correlated

with the number of eosinophils in the peripheral blood. That could explain why the ewes on the machine-milked treatments had lower worm burdens than the twin-suckling ewes, as they had significantly higher eosinophils than the twin-suckling ewes in week seven of lactation. A possible reason for the lower eosinophil number in the twin-suckling ewes and subsequently higher worm burdens may well be the allocation of nutrients to the milk production processes rather than immunity which has been suggested by Houdijk *et al.* (2001a; 2003). Bauman and Currie (1980) suggested that nature has put such a high priority on reproduction that it is allowed to proceed at the expense of other metabolic processes, and as Houdijk *et al.* (2001a; 2003) suggested, at the expense of the immune system as well.

Only blood haematocrit was affected by diet during the current experiment; it was ewes on the HMP dietary treatment that had the higher concentrations. This may be explained by dietary protein as it has been shown to influence haematocrit concentration (Topps and Thompson, 1984). Low haematocrit concentrations coupled with low plasma urea concentrations would suggest a deficiency of dietary protein. In the current experiment, it is likely that the differences in haematocrit were due to the difference in dietary protein. There were no effects of either dietary treatment or milking method on the white blood cell concentrations in the blood. As eosinophils are part of the collection of WBCs, the results may have been expected to reflect the eosinophil results but this was not the case.

5.4.3. Effect of increased MP supply on lymphocyte blastogenesis response of the machine-milked ewes during lactation

Lymphocyte blastogenesis is an accepted means of *in vitro* evaluation of lymphocyte reactivity that has been studied extensively in human and murine systems (Cross *et al.*, 1986). There was an increase in lymphocyte response to the mitogens Con A and PWM throughout lactation in the machine-milked ewes. This temporal increase may have been due to the immediate periparturient period being associated with immunosuppression

(Kehrli *et al.,* 1989). Indeed, Kehrli *et al.* (1989) reported that a lymphocyte response to Con A was decreased in the first week of lactation, which may explain the increase in lymphocyte blastogenesis throughout the six weeks studied in this experiment. Lloyd (1983) found a hyporesponsiveness of lymphocytes to *in vitro* stimulation with mitogens occurred in ewes infected with *H.contortus* at the same time as the PPR. However, in the absence of an uninfected control, it is not possible to attribute a decreased lymphocyte proliferation response to either parasitism or the immunosuppressive effects of parturition.

There were no differences in lymphocyte blastogenesis between the dietary treatments. Protein may not have any beneficial effect on lymphocyte blastogenesis. Coop and Holmes (1996) reported that lymphocyte stimulation to a range of mitogens was generally unaffected by protein supplementation though, in contrast, Burkholder and Swecker (1990), reported an increased blastogenesis reponse to mitogens in protein-deficient animals.

Although lymphocyte blastogenesis is an accepted means of *in vitro* evaluation of lymphocyte activity (Cross *et al.*, 1986), the colorimetric method (Mosmann, 1983) used in this study is less sensitive than the radio isotope labelled thymidine method reported by Pollock (1993). Therefore, subtle differences between treatment groups may have been masked.

5.4.4. Effect of treatment on ewe live weight and body condition score

During pregnancy, ewes offered the HMP treatments gained more weight up to parturition than the ewes offered the LMP treatments ($P < 0.001$). The difficulty of using LW as an assessment of nutrition status during pregnancy is that differences could be attributed to the foetus weights rather than ewe condition. However, at birth, as with Experiments One and Two, there were no differences in lamb birth weight ($P > 0.05$). A more efficient assessment of ewe nutritional status is body condition score (Russel, 1984). However, there were no differences between treatments in body condition scores. After parturition

through until week six of lactation, ewes offered the HMP dietary treatment had a higher LW ($P < 0.05$) and this difference would appear have been carried over from the difference developed through pregnancy.

Machine-milked ewes gained LW during lactation; whilst the twin-suckling ewes lost a significant amount of LW ($P < 0.001$). This appears to reflect the differences in milk yield, where the lower producing machine-milked ewes were provided with an excess of nutrients for the yield they were producing as suggested in Experiments One and Two. By contrast, the higher producing twin-suckling ewes were mobilising body fat for the production of milk regardless of the dietary treatment offered. Again, as during pregnancy, there were no differences in ewe CS which implies that body CS was a less sensitive assessment of body energy reserves. However, as discussed previously, body condition is not an accurate means of assessing energy reserves in the ewe breed used here.

5.4.5. Effect of treatments on ewe plasma metabolites

5.4.5.1. *Indicators of energy status*

The concentrations of plasma βHB may be affected by time of sampling (Topps and Thompson, 1984) and, unlike the previous two experiments, the time of sampling used in the current study was after the longest period of fasting immediately prior to the morning feed, and may therefore provide the most accurate assessment of the energy status of the ewes. All the ewes had βHB concentrations within the acceptable range (i.e. less than 1mmol l^{-1}) which indicates that all the ewes had an acceptable energy intake. During lactation ewes suckling twin lambs had higher βHB levels than machine-milked ewes ($P = 0.034$), and those ewes offered the HMP diets had higher levels than the ewes offered the LMP dietary treatments ($P = 0.049$). This may suggest that the ewes suckling the twin lambs were mobilising more body fat reserves than machine-milked ewes and that ewes on the HMP dietary treatments mobilised more so than those on the LMP dietary treatments as, according to Topps and Thompson (1984), a higher βHB level can be related to the

mobilisation of fat reserves. Ewes suckling twin lambs may have been under a greater nutritional stress due to partitioning of nutrients to the reproduction processes and the greater worm burdens than the milked ewes.

There were no differences in plasma glucose concentrations between treatments. Glucose is not under full homeostatic control so many factors such as time of sampling, hormonal status and stress may influence the results (Topps and Thompson, 1984), and plasma βHB is a more suitable representation of the energy status of the ewes (Russel, 1984).

5.4.5.1. *Indicators of protein status*

Total proteins are the sum of the plasma albumin and the plasma globulin concentrations (Topps and Thompson, 1984). There were no differences between treatments in total protein or globulin concentrations *pre-partum* despite a significant difference in albumin levels – the ewes on the HMP treatment had higher albumin levels than the ewes offered the LMP treatment ($P < 0.001$). Due to the similarities in globulin levels and the differences in albumin levels, it could be presumed that total protein concentration would have been higher in ewes on the HMP treatment, but there was only a slight, not significant difference.

During lactation, ewes offered the HMP treatment had higher albumin ($P < 0.001$) and total protein concentrations ($P = 0.01$) than those offered the LMP treatment, and there were no differences in globulin concentrations. The higher plasma total protein concentrations may be explained by the higher albumin concentrations; however ewes suckling twin lambs had higher total protein concentrations ($P = 0.037$) than machine-milked ewes and this cannot be explained so easily. Had the plasma globulins been higher, it would have been plausible to suggest that the ewes were producing more immunoglobulins against the larger worm burdens, however, this was not the case. During lactation, globulin concentrations were marginally higher in the animals with the higher

total proteins and it is a possibility that globulins had a small contribution to the differences in total proteins levels.

Ewes offered the HMP treatments both *pre-* and *post-partum* had higher ($P < 0.001$) plasma urea levels. This has been discussed in depth in Experiment One and is likely to be the result of the differences in the protein fed in the two dietary treatments.

5.4.6. Effect of treatments on ewe milk production

It was estimated that the ewes suckling twin lambs produced 28% higher daily milk yields than the machine-milked ewes, with an average of 2227ml compared to 1605ml, respectively ($P = 0.001$) and demonstrated a gradual increase from week one of lactation through to week six, whereas the machine-milked ewes had had a reduction in yields (Figure 5.7.). Cant *et al.* (2001) described that ewes rearing twin lambs produced more milk than machine-milked ewes during the first 30 days of lactation. Treacher and Caja (2002) and Cant *et al.* (2001) suggested that a drop in yield at weaning is a common phenomenon and indicates that some aspect of suckling is a stronger stimulator of milk production than machine milking. Mills (1989) and Marnet *et al.* (1998) also suggested that the rupture of the dam-offspring bond can cause a reduction in milk yields. The machine-milked ewes in the current experiment and the previous two experiments had their lambs removed between 48 and 72 hours of birth to minimise the stress, Brandano *et al.* (2002) suggested removing the lamb within 48 hours to prevent a bond developing. It is more likely that it was the reduced frequency of machine milking compared to a lamb suckling between 6 and 12 times a day that had the most influence on milk yield (Cant *et al.*, 2001).

During week one of lactation, the ewes suckling twin lambs that were offered the high MP diet had the highest yield of 2189ml compared to 1715, 1888 and 1754ml for the LMP-S, LMP-M and HMP-M, respectively, and maintained the highest yields throughout lactation. Treacher and Caja (2002) suggested that in early lactation, when energy requirements were

high and voluntary feed intake has not reached its peak, then protein intake was likely to have a critical effect on milk production.

Contrary to the findings of Broquier and Caja (2004) that increased dietary protein and work by Lynch *et al.* (1991) where rumen protected methionine and lysine increased milk yield, there was no significant difference in milk yield between the high and low protein groups in the current experiment. As a consequence of the lack of differences in milk yield, there should have been no differences in fat or protein concentration as there is no difference in the dilution. The milk protein level was lower in the HMP-M treated ewes than the LMP-M treated ewes ($P < 0.05$), which is contrary to expectations as milk protein should positively correlate with an increase in dietary protein (Bocquier and Caja, 2004).

5.5. *Conclusions*

In conclusion, supplementation of the periparturient ewe diet with additional metabolisable protein reduced the FECs of ewes when under a nutritional stress such as rearing twin lambs. Although it appears an expensive alternative to chemoprophylaxis, this may be outweighed by the need to reduce chemoprophylaxis due to nematode resistance, and that lambs from supplemented ewes should grow faster due to better milk production and less contaminated pastures.

Prior to this experiment, it was thought that the use of dairy ewes would allow parameters to be measured to predict the causes and effects of the periparturient rise in FECs to be studied more accurately. However, it can be concluded that the machine-milked dairy ewe is not a suitable model for parasitological research.

CHAPTER SIX – GENERAL DISCUSSION

6.1. Nutrition and nematode infection in sheep

It was established more than 60 years ago that nutrition had an influence on nematode burden (Whitlock *et al.*, 1943). There has also been evidence that protein may have beneficial influence on FECs for many years, for example, Bawden (1969), discovered that young sheep on a low protein diet were more susceptible to infection with *Oesophagostomum columbianum* than those on a high protein plane of nutrition. Recently this discovery has become more important since the emergence of nematodes becoming increasingly resistant to the few classes of chemical anthelmintics available, with little sign of the development of any new chemicals in the near future (Hennessy, 1997a; Waller, 1999). In the last two decades, there have been numerous studies on protein and its effects on nematode infection in lambs (Abbott *et al.,* 1985; 1988; Bown *et al.*, 1991a; Kambara *et al.,* 1993; Israf *et al.,* 1996; Niezen *et al.*, 1998a; 1998b; Datta *et al.,* 1999), and the periparturient female (Donaldson *et al.*, 1998; 2001; Etter *et al.*, 2000; Houdijk *et al.*, 2000a; 2000b; 2001c; 2002; 2003), all of which demonstrating to varying degrees, some beneficial effect of protein on the FECs and worm burdens in these animals. However, the beneficial effects of MP supply on nematode burdens of periparturient ewes have not always been observed (Houdijk *et al.*, 2000c; 2001b; Kahn *et al.,* 2003a). The lack of beneficial effects from increased MP supply in the studies of Kahn *et al.*, (2003a) may have been due to the use of single lamb-rearing ewes. Donaldson *et al.* (1998, 2001) suggested that the PPR is most apparent in ewes rearing twins rather than single lambs.

6.2. Effect of dietary treatments on FECs

The presence of natural adult burdens in Experiments One and Two may have affected the FECs but increased dietary protein has been known to reduce FECs from animals with natural burdens (Coop and Holmes, 1996), therefore it may still have been reasonable to

have expected a reduction of FECs due to increased dietary MP. It may also have been impossible to determine any effect of increased MP on FECs from the machine-milked ewes; the FECs were low from these ewes throughout lactation possibly due to the 'low' milk yields produced by them. Only ewes which suckled twin lambs in Experiment Three demonstrated that the increased MP supply caused a lower faecal egg output when compared to the suckled ewes on the low MP diet. This is in accordance with the findings of Donaldson *et al.* (1998; 2001), Etter *et al.* (2000) and Houdijk *et al.* (2000a; 2000b; 2001c; 2002; 2003), and disagrees with Houdijk *et al.* (2000c; 2001b) and (Kahn *et al.*, 2003a) who reported no beneficial effects of increased MP supply on the PPR.

6.3. Time of onset of the PPR and resistance against established adult nematodes

There was evidence, from the presence of eggs in the faeces of the ewes, that the resistance to the establishment of infection was incomplete during the *pre-partum* period in Experiment Three and possibly Experiment Two although this cannot be confirmed due to the presence of eggs in the faeces at the start of the latter experiment. There was also evidence of an inability to reduce the faecal egg output from an established adult worm burden during the immediate peripartum period in Experiment One prior to challenge commencement in week one *post-partum* and in the unchallenged ewes in Experiment Two throughout pregnancy. This is in contrast to the findings of Leyva *et al.* (1982) who did not observe eggs during pregnancy in sheep infected with 4000 *T. circumcincta* larvae per day from 6 weeks *pre-partum*. O'Sullivan and Donald (1970), Jackson *et al.* (1988), Coop *et al.* (1990) and McAnulty *et al.* (2001) also found that establishment of *T.circumcincta* larvae can occur during pregnancy.

In the present study, particularly from Experiments One and Two, there is evidence that there is an inability to reduce the fecundity of established adult nematodes during the *pre-partum* period especially in the absence of incoming infective larvae. This, however, was discovered because of the possible anthelmintic-resistant nematodes surviving and

subsequently the presence of adult nematodes prior to challenge commencing (section 4.4.1.). Further work on natural adult nematode burdens during pregnancy would need to be performed to confirm that the pregnant ewe is unable to control the fecundity of adult nematodes.

6.4. Effect of treatments on the ewe immunological status

Increased MP supply had no effects on the immunological parameters measured in any of the three experiments undertaken in this study. There were no differences in immunoglobulin levels in Experiment One, peripheral eosinophil numbers in all three experiments and MMCs and mucosal eosinophils in Experiments Two and Three. There were higher MMCs and mucosal eosinophils in the challenged ewes compared to the un-challenged ewes in Experiment Two but this was not influenced by MP supply and was a reaction to the incoming infective stage larvae (Meeusen, 1999; Balic *et al.* 2002). The lack of effects of increased MP supply on the immunological status of the periparturient ewe could suggest that the beneficial effects of increased protein demonstrated in Experiment Three may be due to a direct effect on the nematodes at gut level rather than by re-establishing the ewe's immunity or that another aspect of the immune system, not measured here, is important in the expulsion of nematodes and benefits from increased dietary protein.

6.5. Effects of challenge and dietary treatments on ewe peripheral eosinophils

Peripheral and tissue eosinophilia has often been shown to be an indicator of helminthiasis, suggesting a role for eosinophils in host resistance (Dawkins *et al.*, 1989, Huntley *et al.*, 1995), and reduced dietary protein has been associated with diminished peripheral eosinophil counts in pigs infected with *Trichuris suis* (Pedersen *et al.*, 2002). Charter *et al.* (2000) found higher eosinophil counts in goats on high protein diets compared to those on

low protein diets. The present study has provided no evidence of peripheral eosinophil numbers being affected by the protein level of the diets.

There was, however, a noticeable similarity between all three experiments; regardless of the start of challenge, the eosinophil counts increased after week one of lactation peaking at week three for the machine-milked ewes and to a lesser extent for the suckled ewes (Figure 6.1.). This could indicate that the ewes had a re-establishment of immunity which coincided with the time that a reduction in milk yields and FECs were observed.

The rise in eosinophils could be explained in Experiment one as the increase in eosinophils coincided with the start of challenge in week one of lactation. Pfeffer *et al.* (1996) suggested that previously exposed sheep would have an increase in eosinophils from 7 days post-challenge and Balic *et al.* (2000a) stated that there would be a marked rise in eosinophils in previously exposed animals a few days after challenge. This rise in eosinophils could be as a consequence of the larvae and developing stages becoming embedded in the mucosa (Kimambo *et al.*, 1988).

This does not, however, explain the rise in peripheral eosinophils in Experiments two and three where challenge commenced 3 and 6 weeks *pre-partum,* respectively. The eosinophil counts peaked in week 3 *post-partum* as in experiment 1 (Figure 6.1.). O'Sullivan and Donald (1970) speculated that lactation was responsible for reduced immunity in periparturient ewes. The findings here suggest otherwise and McAnulty *et al.* (2001) suggested that outside the immediate peripartum period, lactation is not inconsistent with strong resistance to larval establishment. It could be argued, however, that three weeks *post-partum* is still in the immediate peripartum period. The rise in eosinophils at this time could be attributed to a re-establishment of immunity in the ewe during lactation as Buddle *et al.* (1992) have suggested that eosinophilia is associated with the expression of resistance to nematodes rather than an indication of the presence of nematodes. Buddle *et al.* (1992) also found that a marked rise in eosinophils in lambs trickle-challenged with *T.*

columbriformis coincided with a decrease in FECs which is also apparent in the current study.

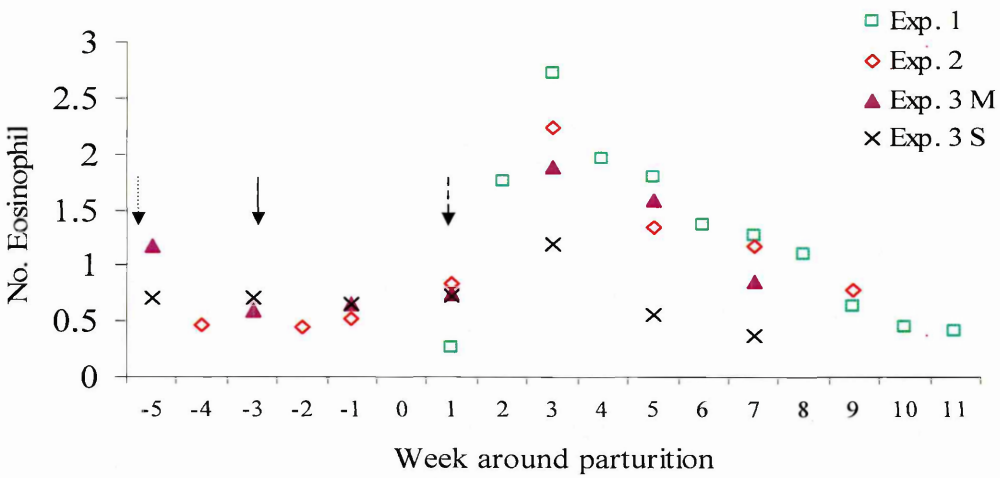


Figure 6.1. *Mean peripheral eosinophil counts (eosinophils x 10^9/ml whole blood) from the three experiments. (Key, M = milked, S = suckled, ⋯→ challenge commenced experiment 3, → challenge commenced experiment 2, - - → challenge commenced experiment 1)*

6.6. The machine milking of ewes and FECs

In the presence of many studies on the relationship between nutrition and the PPR, dairy ewes were used throughout the experiments and were machine-milked for the first two experiments. The use of the dairy ewes would also allow easy quantification of the milk yields produced, rather than estimating milk yield from the growth rate of lambs, and whether there were any effects of the dietary treatments on the yield that could counteract or enhance any beneficial effects on the PPR that arose. It was also suggested that these ewes would be under a greater nutritional pressure than ewes suckling lambs and certainly under more pressure than meat-producing sheep (Thomas, 2003), especially in view of the fact that the ewes were fed diets calculated to AFRC (1993) recommendations, despite the fact that Cannas (2002b) has suggested that AFRC (1993) may have underestimated the nutritional needs of the machine-milked dairy ewe. Chartier *et al.* (2000) found that

higher-yielding goats had higher FECs than the lower-producing ewes, therefore it was presumed the dairy ewes would be affected by the PPR in FECs.

6.7. Milk production

6.7.1. *Increased MP can increase milk yields of milked and suckled ewes*

Experiments one and two demonstrated that an increased MP supply lead to increased milk yields. This was anticipated as it has been observed in cattle (Oldham, 1984), goats (Chartier *et al.,* 2000) and sheep (Brocquier and Caja, 2004; Lynch *et al.*, 1991). Therefore any benefits of the increased production from feeding greater amounts of protein may encourage the adoption of increasing dietary MP supply as a measure nematode control by farmers.

In Experiment three, there was no evidence of an effect of MP supply on milk yields in the machine-milked ewes although it did in the twin-suckling ewes. This provided evidence that ewes suckling twin lambs produce significantly higher yields than those that are machine-milked (Table 6.1). Cant *et al.* (2001) suggested that during the first 30 days of lactation, ewes suckling twins would produce more milk than entirely machine-milked ewes and Treacher and Caja (2002) and McKusick *et al.* (2001) suggested that a drop in yield at weaning is a common phenomenon.

Table 6.1. *The effect of dietary protein on milk yields throughout the three experiments*

	LMP (BMP Exp.2) (ml day^{-1})	HMP (ml day^{-1})	s.e.d.	F prob
Experiment 1	1056	1560	212.6	**
Exp.2 infected	1652	2236	334.7	ns
Exp.2 non inf.	1688	2406	278.5	*
Exp.3 milked	1599	1610	274.3	ns
Exp.3 suckled	1957	2496	236.9	*

Key: ns = non-significant, * = $P < 0.05$, **= $P < 0.01$.

6.7.2. *Overall milk yields*

The daily milk yields from the machine-milked ewes in the three experiments did not attain full potential which should have been in the region of 2.5 litres day^{-1}. The findings from the third experiment indicate that some aspect of lambs suckling is a stronger stimulator of milk production than machine milking – perhaps the fact that lambs feed 6 – 12 times per day (Cant *et al.*, 2001).

The ewes in Experiment one were all challenged with *T. circumcincta* infective larvae during lactation therefore it was difficult to identify the cause of the low yields, whether diet, challenge or the low milking frequency. In early lactation, it is likely that less frequent milking would cause a significant reduction in milk yields (McKusick *et al.*, 2001) although in late lactation, milking frequency would have less of a contribution on yield production (McKusick *et al.*, 2002). Labussiere (1988) described conflicting views on the benefits of 3 times-a-day milking with ewes. The majority found anything from 4% to 58% increase in yield compared to milking twice a day. Increasing the milking frequency in Experiment two, to 3 times a day, along with altering the diet to provide un-ground hay (which would provide longer consumption time which would maintain a correct rumen Ph (Treacher and Caja, 2002)),caused an increase in milk yields.

6.7.3. *Effect of nematode infection on milk yield*

In Experiment Three, despite the fact that milking frequency and diet remained the same as in Experiment Two, the yields were as low as in Experiment One for the ewes on the HMP-M treatment. These ewes experienced a higher intake of larvae (4300 larvae day^{-1} compared to 2000 larvae day^{-1} in Experiments One and Two) so challenge could have affected the yields. Leyva *et al.* (1982) found that daily intakes of 4000 *T. circumcincta* larvae could cause a reduction in daily milk yield and a more rapid decline in yield over time. Vercruysse and Claerebout (2001) and Bliss and Todd (1976) have suggested that sub-clinical nematode infections in lactating cattle have been associated with decreased

levels of milk production. It was anticipated that Experiment Two would have highlighted the effects of worm burden on production, however this was not identified despite there being challenged and non-challenged treatments. It would therefore be impossible to implicate larval challenge as the exclusive reason for the low milk yields in any of the experiments.

6.7.4. *Increased production can lead to greater FECs and nematode burdens*

The results from Experiment Three are in agreement with Donaldson *et al.* (1998; 2001) which indicated that ewes rearing twins had a greater PPR than those rearing single lambs. It was hypothesised that production pressure has an involvement in the PPR. This is also in agreement with Chartier *et al.* (1997; 2000) who noted that high-producing dairy goats were less resistant and/or resilient than their lower-producing counterparts, to experimental nematode challenge. The findings of the final experiment also suggested that increased protein has a beneficial influence on the FECs provided that the ewe is under a high production pressure. Without a high level of production, the plane of nutrition necessary to help reduce FECs in the periparturient ewe is met at feed levels developed by the AFRC (1993) and therefore there is no evidence of effects of MP on the PPR.

6.8. *Conclusions*

By supplementing the periparturient ewe's diet with increased metabolisable protein the faecal egg output of pregnant ewes and ewes suckling twin lambs can be reduced. Machine-milked ewes, however, did not appear to benefit from the increased MP supply apart from an increase in milk yield which was not produced at the expense of the ewe's immunity against nematodes. Either the machine-milked dairy ewe may not be a suitable model for parasitological research, or some un-explained mechanism within the machine-milked ewe may be responsible for the reduced nematode egg output.

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Plasma/Serum Pepsinogen Assay

Materials:

1% Bovine serum albumin in distilled water (BSA)
Glycine buffer 0.1M pH 2, (7.5g glycine + 5.85g Na Cl per litre of
1N Sodium hydroxide 40g/l
10% Trichloracetic acid (TCA)
Folins Reagent (1 part stock Folins:2 parts water)
Stock tyrosine solution. (0.0362g tyrosine in 100 mls 0.1N HCl.
Working standard is a 1:10 dilution of stock with distilled water.
96 well microtitre plate
Small glass filter funnels
Whatman no 6 filter paper
Titertek multiscan ELISA reader 680nm filter

Procedure:

1. Mix glycine buffer and BSA in a ratio of 4:1.
2. Add 200 μl serum or plasma, mix with 1 ml glycine/BSA substrate, incubate at 37°C for 4 hours.
3. To a further 200 μl serum sample add 1 ml glycine/BSA substrate followed immediately by 0.8 mls TCA to precipitate proteins present (this sample acts as an unincubated control).
3. Following incubation add 800 μls TCA to the sample.
4. Both samples can then be filtered through Whatman no 6 filter paper to remove the precipitate (NB centrifugation alone is often not sufficient since some precipitate may become trapped in the meniscus).
5. Pipette 100 μl of each filtered sample into two wells in the microtitre plate and add 100 μl sodium hydroxide and 5μl Folins reagent.
6. The resulting blue colour is fully developed after 3 minutes and is stable for a further 20 minutes.
7. A standard curve is constructed by making up 100μl, 250μl, 500μl, 750μl and 1000μl volumes of working standard to a total volume of 2ml using 1 ml of TCA and distilled water. 100 μl of these standards is added to each of two wells together with 100 μl sodium hydroxide and 5 μl Folins reagent.
8. Plates are read on a Titertek multiscan ELISA reader using a 680nm filter.
9. Calculate serum pepsinogen value from serum pepsinogen data

$$\text{Serum pepsinogen} = \frac{(T-C)}{S-B} \times \mu \times \frac{1000}{V} \times \frac{1}{I} \times 1000$$

Where T = Test value, C = Control value, S = Standard value, μ = μmol tyrosine in standard, V = Volume of serum, I = Incubation time (minutes).
Using the centre standard which has 0.005 μmoles tyrosine and 0.01ml serum then serum pepsinogen value (SPV) is calculated as follows

$$\text{SPV} = \frac{(T-C)}{(S-B)} \times 2083 \text{ mU tyrosine per litre per minute}$$